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18. Studies on Protein-Sulfhydryl Reagents. I.[*3] Synthesis of Benzimidazole Derivatives of Maleimide; Fluorescent Labeling of Maleimide.

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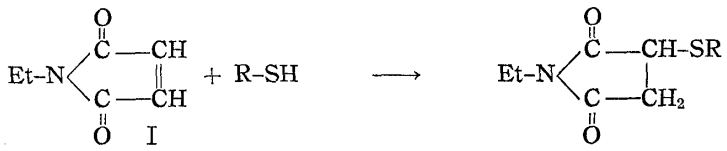
Sulfhydryl groups in protein play a significant role in biological systems and have been the subject of extensive studies in protein chemistry.[1,2] Many "sulfhydryl reagents" have been thus developed and used in the field of enzymatic research and structural studies on protein. N-Ethylmaleimide (I, herein abbreviated as NEM) is particularly favored since this reagent reacts rapidly and specifically with these sulfhydryl groups to give addition products under mild conditions.[3]



C^{14}-Labeled NEM was successfully used in the structural study of myosin-ATPase to chase peptide fragments containing the active site which was assumed to involve sulfhydryl groups.[4] A colored reagent of NEM derivative, N-dimethylaminodinitrophenylmaleimide (DDPM), was also synthesized[5,6] and its reaction with cysteine residue in albumin was studied.[6] The applicability of the colored reagent is, however, still limited because it has low solubility and coloration itself is not always intense enough. C^{14}-Labeled reagents are rather expensive. Thus, finding some practical labeling technique seemed promising for wide-spread application.

If some appropriate group, which exhibits fluorescence of sufficient intensity in the range of visible wave length under ultraviolet light, can be introduced in NEM molecule

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without changing its chemical reactivity, such a compound would be conveniently used as a reagent in dealing with proteins containing sulfhydryl groups. This simple way of "labeling" could conceivably substitute a conventional but expensive radioactive one to some extent and may be characterized as "fluorescent labeling." Fluorescent compounds have been employed as useful tracer mainly in the field of immunological study. Some artificial fluorescent conjugates of proteins were prepared for that purpose.[7,8] In general paper-chromatographic procedure, detection of spots under ultraviolet light is a widely used technique.[9] There have been, however, not ample precedents in which a special chemical reagent was labeled with a chemical group as a fluorophore to trace some specified organic reaction.

Substituted benzimidazoles were selected for our initial test to find labeling group. None of 5(or 6)-methoxy- (Ⅱb), 5,6-dimethoxy- (Ⅱc), and simple benzimidazole showed significant fluorescence under practical conditions. 4(or 7)-Methoxybenzimidazole (Ⅱa), however, was found to exhibit fairly strong fluorescence under ultraviolet light. This finding led us to attempt to combine 4(or 7)-methoxybenzimidazole moiety as a fluorophore with maleimide moiety as a reactive component. In this work, N-[(4(or 7)-methoxy-2-benzimidazolyl)methyl]maleimide (Ⅲa) was synthesized in the purpose of designing a novel labeling reagent. In order to prepare various N-substituted maleimides along this line, a suitable synthetic route involving the cyclization of maleamic acids (Ⅳ) to maleimides (Ⅲ) was explored. Thus, polyphosphoric acid (PPA) was successfully used in the maleimide cyclization and demonstrated to be a general reagent for this reaction.

Benzimidazoles (Ⅱa~d) were synthesized from the corresponding *o*-phenylenediamines (Ⅴa~d) by Phillips' method.[10,11] Synthesis of Ⅵa and Ⅶa were described in the literature.[12] 2,3-Diaminoanisole (Ⅴa) was fused with hippuric acid to give Ⅵb, which was hydrolyzed to the amine (Ⅶb) hydrochloride. Since the amines (Ⅶa~b) were highly soluble in water, separation of free base (Ⅶa) from a reaction mixture was achieved using column chromatography of DEAE-cellulose. The amines were combined with maleic anhydride in an inert solvent at room temperature in good yields.[13,14]

The cyclization of maleamic acid has been studied by many groups. Simple fusion,[15] heating in solvents,[16] treatment with dehydrating agent such as acetic anhydride.[13,17,18] trifloroacetic anhydride[19] or phosphorus pentoxide[14,20,21] have been described. When substituted maleamic acids contain any sensitive group to a dehydration agent of acylating type, or have high melting point or low solubility, however, these known methods can not work in each of such restricted synthetic condition. Benzimidazole derivatives are obviously the case.

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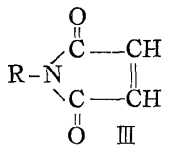

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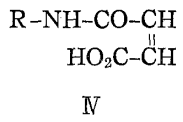

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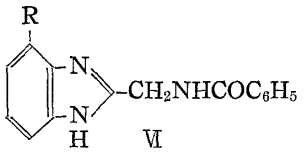
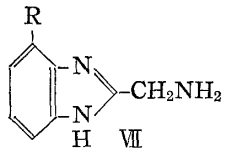


Ⅵ a : R=H b : R=MeO



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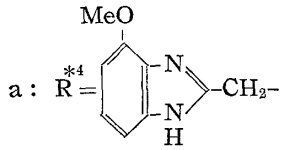
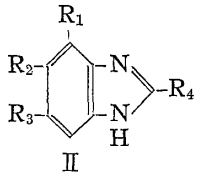


a : R=*4 b : R=

c : R=benzyl d : R=butyl e : R=phenyl



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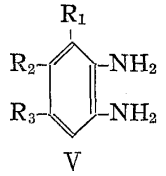

Ⅴ

a : R_1=MeO, R_2=R_3=R_4=H b : R_1=R_3=R_4=H, R_2=MeO
c : R_1=R_4=H, R_2=R_3=MeO d : R_1=MeO, R_2=R_3=H, R_4=Me

Heating maleamic acids (Ⅳa~e) in excess amount of PPA around 120° for 20 minutes effected smooth cyclization with moderate yields thus offering a favorable general method. Maleimides (Ⅲa~e) prepared in this manner are listed in the Table I.

Table I. Imide Cyclization

Material (g.)	PPA (g.)	Temp. (°C)	Time[a] (min.)	Product	Yield[b] (%)	m.p. (°C)	Recryst. solvent
Ⅳa, 0.5	2.0	115~120	20	Ⅲa	65	168.5~169.5	benzene
Ⅳb, 0.25	0.75	100	10	Ⅲb	65	180~181	MeOH
Ⅳc, 2.0	6.0	140	20	Ⅲc	20	69~70[c]	hexane
Ⅳd, 2.5	7.5	120	20	Ⅲd	21	86~89/15 mm.[d]	
Ⅳe, 1.0	3.0	120	20	Ⅲe	30	87~89[e]	H_2O
Ⅷa, 0.5	1.5	120	20	Ⅸa	44	101~103[f]	EtOH
Ⅷb, 2.0	6.0	120	20	Ⅸb	49	153.5~154.5[g]	〃
Ⅹ, 2.5	7.0	120	20	Ⅺ	26	115~116.5[h]	〃

a) heating time after clear solution resulted
b) yields of recrystallized products
c) ref. 15), 69~70°
d) ref. 16), b.p_{20} 103~104°
e) ref. 22), 90~91°
f) ref. 23), 103°
g) ref. 24), 155°
h) ref. 25), 115~116°

N-Benzyl- or phenyl-succinamic acid (Ⅷa or b) and N-benzylphthalamic acid (Ⅹ) also gave the corresponding succinimide (Ⅸa or b) and phthalimide (Ⅺ) as shown in the Table. This fact indicates that the condition may also provide a general imide synthesis not limited to maleimide cyclization. Treatment of Ⅳc with dicyclohexylcarbodiimide resulted in the formation of the isoimide (Ⅻ), the structure of which was assigned based on the infrared spectrum. This effect of DCC was in accordance with the observation in the literature.[26]

*4 Only one of two possible alternative structures of benzimidazoles is shown in this paper.

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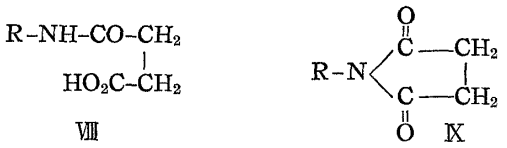

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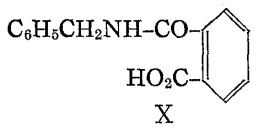
a : R=benzyl b : R=phenyl

Ⅸ



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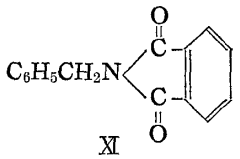

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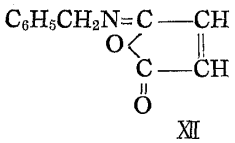

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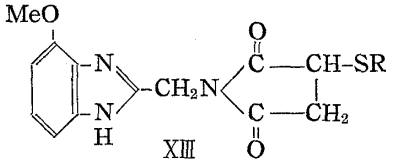

ⅩⅢ

These benzimidazole derivatives of maleimide (Ⅲa~b) and (Ⅲc) reacted rapidly with sulfhydryl group of cysteine or 2-mercaptoethanol in weak alkaline medium and the reaction could be checked by measuring the decrease in absorbancy around 300 mμ, which gave maximal difference before and after the addition of sulfhydryl compounds, as illustrated in Figs. 1 and 2. Fig. 3 shows ultraviolet spectrum of Ⅲa.

Fluorescence characteristics of substituted benzimidazoles are shown in the Table Ⅱ. Except for Ⅲa, 4(or 7)-methoxy derivatives of benzimidazole exhibited fairly intense fluorescence, and their minimum detectable amounts in the spot on paper under ordinary conditions were small enough for labeling purpose. The reagent (Ⅲa) itself showed no fluorescence, but appeared as absorbing spots on paper. After reaction with sulfhydryl compound, however, expected product (ⅩⅢ) again brought about fairly strong emission, intensity of which was about 4 times as much as that of coloration developed by DDPM in visual detection. Their fluorescence spectra are shown in Fig. 4.

Table Ⅱ. Emission Maxima of Benzimidazole Derivatives and their Minimum Detectable Amount

Compd.	Max. No.	λ_{ex}^{max} a) (mμ)	λ_{em}^{max} a) (mμ)	Minimum detectable amount (μμ*M*) c)	
				at 2536 Å	at 3650 Å
Ⅱa	1	298	438	0.08	1.0
	2	415	(475) b)		
Ⅱd		295	438	0.12	1.0
Ⅶb	1	427	522	0.14	0.27
	2	317	480		
Ⅲa		403	(482) b)	120 (0.8) d)	
ⅩⅢ		306	475	0.24	
DDPM				1.0 (yellow color) e)	

a) See the experimental.
b) Fluorescence is extremely faint.
c) The minimum detectable amount was determined on filter paper (Toyo-Roshi No. 51-A) in 5 mm.-diameter spot under UV light by MANASURU LIGHT.
d) absortion on paper as dark violet spot
e) detection by color

An electron of the unshared electron pair on the nitrogen atom(s) in heterocyclic rings undergoes a n-π^* transition according to Kasha.[27] This excited state in turn undergoes a radiationless transition to the triplet state; consequently such compounds do not exhibit fluorescence, *i.e.*, inner quenching is observed. When the unshared

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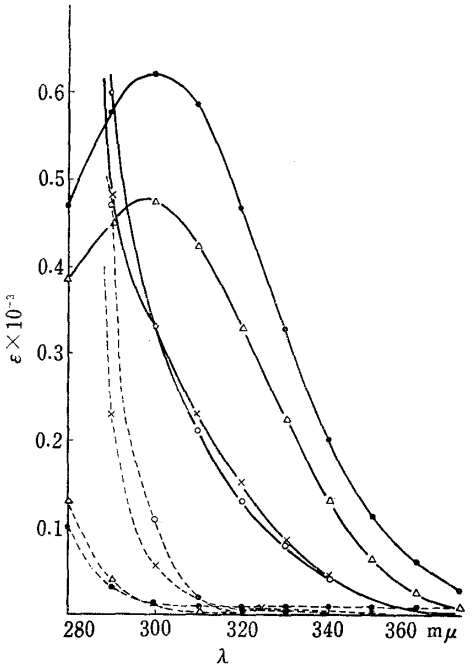

Fig. 1. Ultraviolet Spectra of Benzimidazole Derivatives of Maleimide

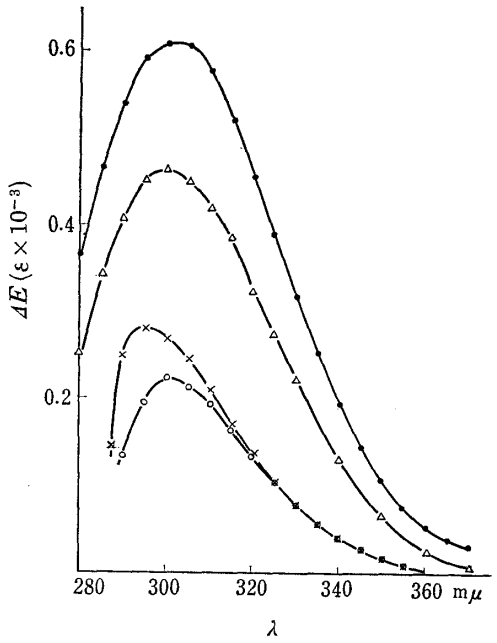

Fig. 2. Differential Spectra between Maleimide Derivatives and their S-Adducts

●—● I, ○—○ IIIa, ×—× IIIb, △—△ IIIc
before (—) and after (---) the addition of 2-mercaptoethanol

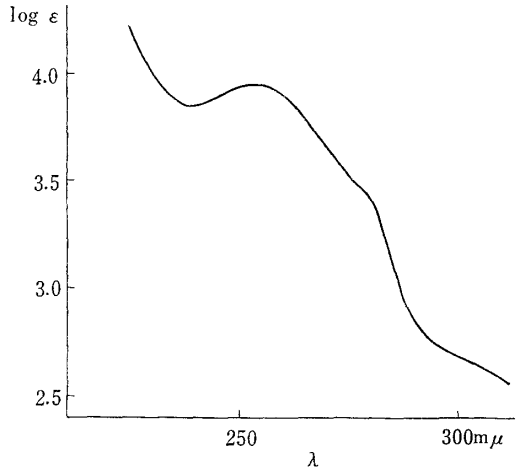

Fig. 3. Ultraviolet Spectrum of IIIa in Ethanol

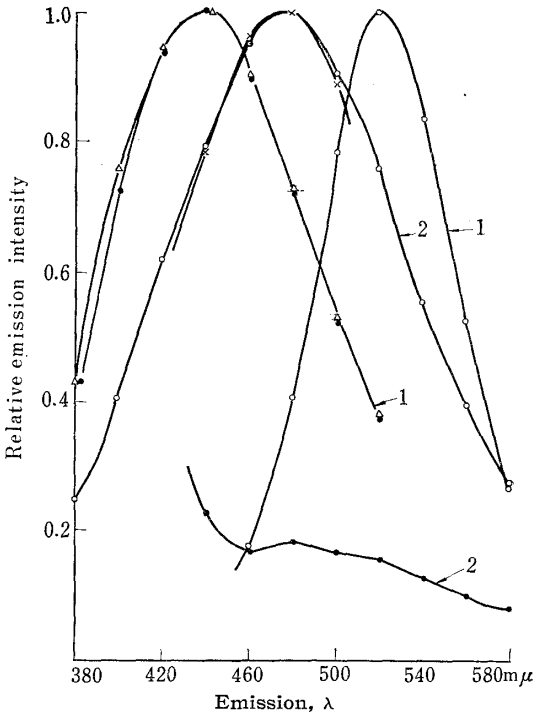

Fig. 4. Fluorescence Spectra of Benzimidazole Derivatives

●—● IIa △—△ IId, ○—○ VIIb, ×—× XIII

Spectra were determined at each λ_{ex}^{max}, shown as 1 or 2, which were described in Table II. Intensity is expressed relatively, taking the maximal one as 1.0 in each derivative. In the case of IIa-2, intensity was taken arbitrarily.

electron on the nitrogen atom becomes involved in protonation in acid medium, transition as above becomes less likely or non-existent and hence enhanced fluorescence is observed.[28,29] This interpretation explains the marked increase in fluorescence intensity of these benzimidazoles in acid medium. The electronic excited state of maleimide moiety of IIIa may be assumed to be leveled closely below that of benzimidazole moiety. This situation might lead to energy transfer between these two excited states. The observed change of fluorescence in going from IIIa to XIII is possibly explained by this assumed internal energy transition.

The study of application of IIIa as protein-sulfhydryl reagent is in progress.

Experimental*5

4(or 7)-Methoxybenzimidazole (IIa)——2,3-Diaminoanisole (Va)[30,31] was obtained by catalytic hydrogenation of 2,3-dinitroanisole[32] with PtO_2 in ethyl acetate solution. IIa was prepared from Va by Phillips' method. Colorless needles from H_2O, m.p. 169~170° (lit.[33] 168°).

5(or 6)-Methoxybenzimidazole (IIb)——3-Nitro-*p*-anisidine was hydrogenated to give 3,4-diaminoanisole (Vb),[34] which was reacted as above to produce (IIb). Picrate, yellow needles from EtOH, m.p. 190~191° (lit.[35], 191°). Hydrochloride, colorless needles from EtOH-EtOAc, m.p. 199~202°.

5,6-Dimethoxybenzimidazole (IIc)——4,5-Dinitroveratrole was hydrogenated to give 4,5-diaminoveratrole (Vc). A mixture of crude Vc (1.5 g.), 80% formic acid (0.79 g.) and 4*N* HCl (7.5 ml.) was refluxed for 30 min. at 130~140° (bath temp.). After cooling, colored precipitate was collected, suspended in H_2O and made alkaline with 10% NH_3, saturated with K_2CO_3 and extracted with AcOEt. Solvent was removed *in vacuo* and the residue was recrystallized from AcOEt to give slightly brown needles, m.p. 182°; yield, 35%. *Anal.* Calcd. for $C_9H_{10}O_2N_2$ (IIc): C, 60.66; H, 5.66; N, 15.72. Found: C, 60.47; H, 5.64; N, 15.76. Hydrochloride, fine crystals from MeOH of m.p. 277° (decomp.).

2-Methyl-4(or 7)-methoxybenzimidazole (IId)——Prepared by Phillips' method, m.p. 163~164° (lit.[36] 163°).

2-Benzamidomethyl-4(or 7)-methoxybenzimidazole (VIb)——The crude amine (Va) from 37 mmoles of 2,3-dinitroanisole was mixed with finely powdered hippuric acid (6.6 g.; equimolar amount) and the mixture was heated in an oil-bath (170~180°) for 20 min. Water was formed and distilled away as condensation went on. After cooling the whole was well triturated with aq. $NaHCO_3$ and AcOEt to be solidified. The solid mass was suction-filtered, washed with H_2O and recrystallized from EtOH yielding almost colorless prisms of m.p. 182~184°, 5.2 g. The second crop (1.0 g.) was obtained from the mother liquor; yield, 51% in total. *Anal.* Calcd. for $C_{16}H_{15}O_2N_3$ (VIb): C, 68.31; H, 5.38; N, 14.94. Found: C, 68.31; H, 5.30; N, 14.93.

2-Aminomethyl-4(or 7)-methoxybenzimidazole (VIIb)——VIb (5.2 g.) was refluxed with 25% HCl (40 ml.) for 2 hr. The cooled solution was filtered to remove benzoic acid, treated with charcoal and evaporated *in vacuo* to dryness and dried in a vacuum desiccator over KOH. The dried residue on recrystallization from MeOH-AcOEt gave colorless needles of m.p. 276~278° (decomp.), 3.4 g. or 75%. *Anal.* Calcd. for $C_9H_{11}ON_3$-2HCl (dihydrochloride of VIIb): C, 43.20; H, 5.24. Found: C, 43.09; H, 5.30.

Aqueous solution of the hydrochloride was made alkaline by adding excess of K_2CO_3, the whole was evaporated to dryness *in vacuo* and extracted with tetrahydrofuran. On recrystallization from $CHCl_3$, the residue, obtained by evaporation of the solvent, gave the base as almost colorless needles of m.p. 149~151.5°; 85%. *Anal.* Calcd. for $C_9H_{11}N_3O$ (VIIb): C, 61.00; H, 6.26; N, 23.72. Found: C, 60.84; H, 6.19; N, 23.64.

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*5 All melting points are uncorrected.

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2-Aminomethylbenzimidazole (VIIa)——The hydrochloride was prepared essentially following the lit.[12] Colorless fibres from MeOH-AcOEt, m.p. 263~265° (decomp.) (lit.[12,37] 263° (decomp.)). The free base was found to be highly soluble in H_2O. Aqueous solution containing the hydrochloride (0.5 g.) was eluted through the column of DEAE-cellulose (12.0 g.) with H_2O (100 ml.). The free base (VIIa) was obtained on evaporation of the elute *in vacuo* as a viscous oil; 0.22 g. or 59%.

Maleamic Acids : General Procedure——To a solution of maleic anhydride in $CHCl_3$ or tetrahydrofuran is added a solution of equimolar amount of amine in the same solvent at a room temperature under water-cooling. Normally exothermic reaction takes place and maleamic acid begins to separate, which is collected and recrystallized (usually, EtOH or $CHCl_3$). Crude yields are nearly quantitative.

N-[(4(or 7)-Methoxy-2-benzimidazolyl)methyl]maleamic Acid (IVa)——Crude product was collected from tetrahydrofuran solution in 90% yield. Recrystallization from H_2O gave crystalline fine powder of m.p. 181~183°. This sample, after drying in vacuum over P_2O_5 at a room temperature overnight, had the same melting point and showed some hydrated results on analysis. At an elevated temperature on drying, it seemed to lose combined H_2O gradually, showing melting point of wide range. After drying at 60~70° over P_2O_5 for 24 hr., it had m.p. of 123~125°, and was found to be dehydrated by analysis. *Anal.* Calcd. for $C_{13}H_{13}O_4N_3$ (IVa) : C, 56.72; H, 4.76; N, 15.27. Found : C, 56.63; H, 4.87; N, 15.38.

N-(2-Benzimidazolyl)maleamic Acid (IVb)——Crude product of m.p. 132~132.5° deposited from water, presumably having some H_2O of crystallization. Recrystallization from EtOH gave colorless needles of m.p. 161~162.5°. *Anal.* Calcd. for $C_{12}H_{11}N_3O_3$ (IVb) : C, 58.78; H, 4.52; N, 17.13. Found : C, 58.59; H, 4.51; N, 17.23.

Maleimide Cyclization : General Procedure——Maleamic acid (1 part) was mixed up with PPA (3~4 parts) and the mixture was heated at 100~120° until a clear solution resulted (ca. 10 min.). This was further heated at around 120° for 10~20 min. After cooling the mass was poured into cracked ice, $CHCl_3$ added and neutralized by adding powdered $NaHCO_3$ and organic layer was separated. The H_2O layer was washed with $CHCl_3$ and combined $CHCl_3$ extract was washed with cold aqueous $NaHCO_3$, saturated NaCl solution and dried over Na_2SO_4. Crude imide was obtained on evaporation of solvent *in vacuo*.

N-[(4(or 7)-Methoxy-2-benzimidazolyl)methyl]maleimide (IIIa)——IR ν_{max}^{Nujol} cm^{-1} : 1775 (w), 1716 (s) (C=O, maleimide). UV λ_{max}^{EtOH} mμ (log ε) : 251 (3.95), 280 (3.44); 300 (2.69) (maleimide absorption). *Anal.* Calcd. for $C_{13}H_{11}O_3N_3$ (IIIa) : C, 60.70; H, 4.31; N, 16.34. Found : C, 60.89; H, 4.46; N, 16.26.

N-[(2-Benzimidazolyl)methyl]maleimide (IIIb)——IR ν_{max}^{Nujol} cm^{-1} : 1780 (w), 1715 (broad) (C=O, maleimide). UV : λ_{max}^{EtOH} mμ : 244, 276, 283 (maleimide absorption). *Anal.* Calcd. for $C_{12}H_9O_2N_3$ (IIIb): C, 63.43; H, 3.99; N, 18.49. Found : C, 63.07; H, 4.40; N, 18.19.

N-Benzylisomaleimide (XII)——A solution of IVc (1.0 g.) in AcOEt was reacted with DCC (1.0 g.) in AcOEt by a dropwise addition. The mixture was stood in a refrigerator for 3 hr. followed by filtration to remove the urea which had separated. Several drops of 30% AcOH were added and the solution was allowed to stand for 2 hr. in a cold to decompose excess of DCC, then washed with aq. $NaHCO_3$, H_2O, dried(Na_2SO_4). The solvent was removed *in vacuo* and the residue was recrystallized from hexane to give colorless feathers of m.p. 47~50°, 390 mg. or 42%. IR ν_{max}^{Nujol} cm^{-1} : 1782 (s), 1700 (s) (C=O, lactone of isomaleimide).[26] *Anal.* Calcd. for $C_{11}H_9O_2N$ (XII) : C, 70.58; H, 4.85; N, 7.48. Found : C, 70.49; H, 4.79; N, 7.54.

Ultraviolet Spectra(Figs. 1, 2)——UV absorption of the maleimide (I) and its benzimidazole derivatives (IIIa, b, c) was determined in weakly alkaline aqueous solution with Hitachi Spectrophotometer EPU-2A (IIIb; in 50% EtOH). 15% Excess of 2-mercaptoethanol was added to each 1 m*M* solution of compounds for the addition reaction, which completed within 1 min.

Fluorescence Spectra (Fig. 4 and Table II)——Adequate amount of sample (free base) was dissolved in aq. EtOH and pH was adjusted around 1~2 with *N* HCl. Fluorescence was determined at the wave length of exciting light (λ_{ex}^{max}), which gives the maximal intensity, with Hitachi Spectrophotometer EPU-2A attached with fluorometric accessary (Xenon lamp). λ_{ex}^{max} and λ_{em}^{max} were determined as follows; at first, the wave length (λ'_{em}) of emission light giving the maximal intensity was determined at an exciting wave length (λ'_{ex}). Then, at λ'_{em}, the maximal exciting wave length (λ''_{ex}) was reexamined and λ''_{em} at λ''_{ex} was rechecked. Such a procedure was repeated until the values were fixed.

Fluorescence of addition product (XIII) of IIIa and 2-mercaptoethanol was observed in the mixture of IIIa and 10% excess of SH compound in aqueous solution containing 0.01*M* phosphate buffer (pH 7.6), acidified with *N* HCl after standing at room temperature for 15 min.

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Summary

N-[(4(or 7)-Methoxy-2-benzimidazolyl)methyl]maleimide (Ⅲa) was synthesized as "fluorescence-labeled" protein-sulfhydryl reagent. Polyphosphoric acid was proposed to be a general reagent for imide cyclization reaction. Fluorescent characteristics of some benzimidazole derivatives were described.

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19. Keiji Sekiguchi, Noboru Obi, and Yoshio Ueda: Studies on Absorption of Eutectic Mixture. II.*1,*2 Absorption of Fused Conglomerates of Chloramphenicol and Urea in Rabbits.

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Adjustment of the absorption rate of some kinds of medicinals is an important problem in order to improve their therapeutic effectiveness. For this purpose, chemical, physical and physiological devices were hitherto employed. Immediate or prolonged action is often achieved by modifying the chemical constitution of the original drug compound. For example, prolonged stay of sulfonamides in the body is accomplished by adding some functional groups to their main structure. Physical techniques applied to preparations, such as coated or multi-layered tablets are also effective for the purpose of obtaining desired therapeutic results. Administration of a physiologically active adjuvant, such as probenecid in penicilline therapy, often changes the duration interval.

In the preceding paper, the authors reported that the eutectic mixture of sulfathiazole and urea showed sooner absorption and higher blood levels of sulfathiazole than the ordinary one or their simple mechanical mixture, when administered by oral route. The method for adjusting the rate of absorption by preparing a fused conglomerate is the one entirely different from those that have been attempted in literature, and is thought to have wide applicability.

In the present paper, the authors investigate some physico-chemical properties of the fused conglomerates of chloramphenicol and urea, and examine the effects of them on the absorption of the antibiotics in rabbits.

Experimental and Results

Materials——Size enlargement of chloramphenicol was done by recrystallizing the commercial product from H_2O.*4 Na-CMC was J.P. grade. Urea and other chemicals used were analytical grade.

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*3 Kita-12-jo, Nishi-5-chome, Sapporo, Hokkaido (関口慶二, 小尾 陞, 上田芳雄).
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