Degradation was observed (Table IV), but no interfering HPLC peaks were present. The HPLC method thus appears to be stability indicating.

Analysis of chlorobutanol by HPLC using UV detection at 210 nm is a reasonable alternative to the GC methods of analysis. Several years ago, the HPLC analysis of 'non-UV absorbers,' such as chlorobutanol, was thought to be difficult if not impossible (20); however, modern UV detectors can readily operate at the lower wavelengths (*i.e.*, 200–220 nm), and the quantitation of 'non-UV absorbers' has now become routine.

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Adjuvant Effects of Glyceryl Esters of Acetoacetic Acid on Rectal Absorption of Insulin and Inulin in Rabbits

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Abstract □ The promoting effect of glyceryl esters of acetoacetic acid on the rectal absorption of insulin and inulin was studied. A decrease in the serum glucose level was observed in rabbits following the administration of an insulin suppository containing glyceryl-1,3-diacetoacetate (adjuvant II) or 1,2-isopropylideneglycerine-3-acetoacetate (adjuvant IV). The promoting effects of adjuvants II and IV on the rectal absorption of insulin and inulin were suppressed by the addition of calcium and magnesium to the suppository. This indicates that adjuvant interaction with the calcium and magnesium ion located in the rectal membrane is involved in the enhanced absorption of insulin and inulin. Adjuvant release from the suppository formulation in addition to adjuvant lipid solubility were found to be other important factors for enhanced absorption of insulin and inulin.

Keyphrases □ Insulin—adjuvant effects of glyceryl esters of acetoacetic acid on rectal absorption, rabbits, inulin □ Inulin—adjuvant effects of glyceryl esters of acetoacetic acid on rectal absorption, rabbits, insulin □ Glyceryl esters—acetoacetic acid, adjuvant effects on rectal absorption of insulin and inulin, rabbits

In a previous paper (1), the effect of enamine derivatives of DL-phenylglycine on the rectal absorption of insulin was reported. It was suggested that rectal absorption of insulin was enhanced due to an interaction between the enamine derivatives and the calcium ions in the membrane. This interaction caused a temporary change in the integrity of the membrane allowing the insulin to pass more easily through the barrier. The active forms of the phenylglycine enamines are considered to be predominantly anionic. The interaction of these adjuvants with calcium and magnesium may be through the carboxylate moiety and/or the enamine moiety.

It has been reported (2) that intravenous administration of acetoacetic acid enhanced the distribution of chloropropamide and sulfadimetoxide to the red blood cells, indicating some change in erythrocyte membrane permeability. In the present paper, the glyceryl esters of acetoacetic acid were examined as adjuvants for promoting the rectal absorption of insulin and inulin. The ability of these nonionic compounds to be released from the suppository formulations, to permeate the rectal membrane, and to interact with divalent metal ions was examined.

EXPERIMENTAL

Materials—Glyceryl esters of acetoacetic acid were routinely synthesized by adding acetoacetic acid to glycerol or 1,2-isopropylideneglycerol in the presence of potassium acetoacetate (a catalyst) at

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Table I—Glyceryl Esters of Acetoacetic Acid

Adjuvants		Physical State	IR, cm^{-1} [a] C=O	IR, cm^{-1} [a] O—H	NMR, ppm
Glyceryl-1-monoacetoacetate	I[b]	Oil	1720, 1705	3400	2.18 (s, 3H, $-CH_3$), 3.39 (b, 2H, $-CH_2OH$), 3.53 (s, 2H, $-COCH_2CO-$), 4.03 (m, 2H, $-CH_2OC-$), 4.30 (b, 1H, $CH-OH$), 4.65 (b, 2H, $-OH$).
Glyceryl-1,3-diacetoacetate	II[b]	Oil	1720, 1705	3450	2.01 (s, 6H, $-CH_3 \times 2$), 3.55 (s, 4H, $-COCH_2CO- \times 2$), 4.15 (s, 4H, $-CH_2O- \times 2$), 4.25 (b, 2H, $CH-OH$).
Glyceryl-1,2,3-triacetoacetate	III[b]	Oil	1722, 1707		2.00 (s, 9H, $-CH_3 \times 3$), 3.55 (s, 6H, $-COCH_2CO- \times 3$), 4.33 (d, J = 6.0Hz, 4H, $-CH_2O- \times 2$), 5.30 (quintet, J = 6.0 Hz, 1H, $-CH-O-$).
1,2-Isopropylidene glyceryl-3-acetoacetate	IV[c]	Oil	1720, 1709		1.28 (s, 3H, $-CH_3$), 1.33 (s, 3H, CH_3). 2.19 (s, 3H, $-CH_3$), 3.37 (s, 2H, $-COCH_2CO-$), 3.70 (m, 2H, H), 4.07 (s, 2H, $-CH_2OC-$), 4.10 (m, 1H, $CH-O$).

[a] Liquid form. [b] Compounds I, II, and III were purified by chromatographic method: silica gel (70 ~ 325 mesh) was the column material; a mixture of benzene and ethylacetoacetate (2:1) was the mobile phase. [c] Compound IV was purified by a vacuum distillation method.

Table II—Suppository Formulations [a]

Formula	Adjuvant (50 mg)	Silicon Dioxide (5 mg)	Insulin (3 IU)	Inulin (25 mg)	Triglyceride Base, q.s.	Calcium Gluconate (7.5 mg)	Magnesium Chloride (3.5 mg)
1	II or III	X	X	—	X	—	—
2	I or IV	—	X	—	X	—	—
3	II or IV	Added only with adjuvant II	X	—	X	X	—
4	II or IV	Added only with adjuvant II	X	—	X	—	X
5	I, II, III or IV	Added only with adjuvants II or III	—	X	X	—	—
6	II or IV	Added only with adjuvant II	—	X	X	X	—
7	II or IV	Added only with adjuvant II	—	X	X	—	X

[a] X indicates compounds present.

100–110° while stirring. The insolubles, if necessary, were removed from the mixture by filtration. The crude products obtained were purified by liquid chromatographic methods (Table I). Commercially available crystalline beef insulin (zinc content 0.5% w/w on dry basis, 24.5 IU/mg) was used throughout the experiments[1]. Other reagents used were of analytical grade.

Preparation of Suppositories—Suppositories were prepared by combining the adjuvant, silicon dioxide[2], and either insulin or inulin with a triglyceride suppository base[3] (melted on a hot plate at 40°) according to the formulas in Table II. Silicon dioxide was added to the suppositories prepared with adjuvants II and III to disperse each adjuvant in the suppository base. The molten liquid was poured into disposable plastic molds[4]. The suppositories were allowed to stand for 2 hr at room temperature and were then stored in a refrigerator until used. Each suppository was slightly conical in shape with a rounded apex, measured 25 mm in length and 7 mm in maximum diameter, and weighed ~1 g.

***In Vivo* Studies**—Male white rabbits (2.5–3.0 kg) were fasted (with water available) for 16 hr prior to experimentation. One-milliliter control blood samples were collected from a marginal vein before the rectal administration of the suppository. After rectal administration of the suppository, the anus was closed with a plastic clip to prevent leakage of the rectal contents during the experiments. Blood samples were collected at specific time intervals for 3 hr. Each sample was frozen until assays of glucose and insulin were made.

***In Vitro* Studies**—Male Sprague-Dawley rats (250–300 g) were fasted (with water available) for 24 hr prior to the experiments. After decapitation and excision of the gut, the rectum and anus were washed with a Krebs-Ringer's solution containing 0.3% glucose (pH 7.4). The rectal segment was ligated at the anus, a 0.1-g liquified suppository was placed

Table III—Control Experiments in Rabbits without Insulin [a]

Triglyceride as Suppository Base	Serum Glucose Level mg/100 ml, 0	0.5	1.0	1.5	2.0	2.5, hr
+ I	108.6 (12.6)	127.4 (6.9)	113.5 (14.8)	120.3 (12.6)	117.2 (18.3)	114.8 (13.9)
+ II	102.9 (10.5)	129.7 (18.9)	124.6 (16.5)	112.8 (5.7)	106.4 (11.7)	109.3 (8.4)
+ III	112.1 (7.3)	119.3 (12.7)	119.5 (17.1)	112.7 (9.3)	117.1 (12.4)	109.7 (9.7)
+ IV	99.7 (4.1)	107.4 (8.5)	103.2 (9.2)	105.1 (9.3)	103.8 (12.4)	108.2 (6.0)
Base alone[b]	102.9 (11.4)	135.8 (21.4)	127.0 (16.3)	119.3 (20.1)	120.0 (8.5)	109.2 (10.2)

[a] Each figure is the mean ± standard deviation of six rabbits. [b] No significant differences in the serum glucose level resulted from suppositories administered with adjuvant compared to suppositories administered without adjuvant.

[1] Common Wealth Serum Laboratories, Australia.
[2] Aerosil 200, Colloidsilica Begussa, Germany.
[3] Witepsol H-15, Chemishe Werk, Witten, Germany.
[4] Nichii Packing Co., Ltd., Osaka, Japan.

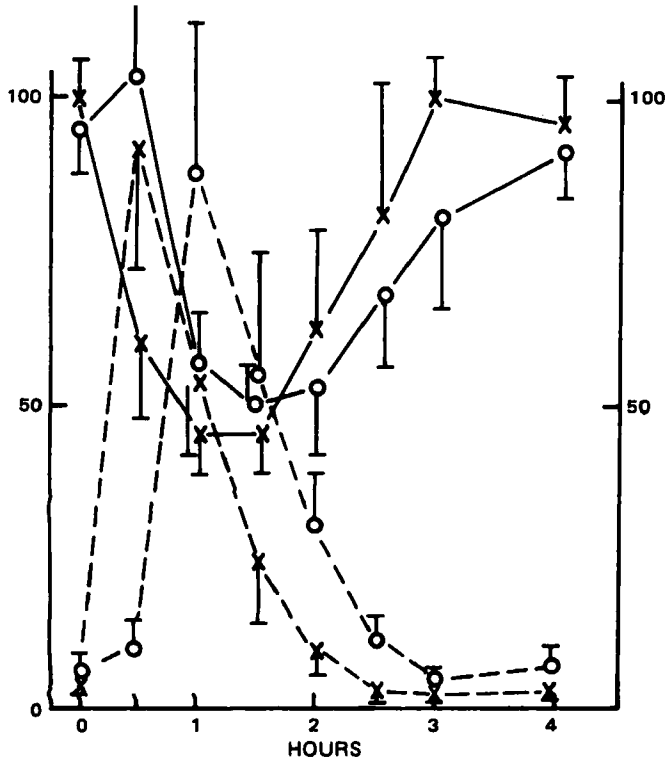

Figure 1—*Changes in serum glucose levels (—) (mg/100 ml) and serum insulin levels (---) (μIU/ml) in rabbits after administration of insulin suppositories containing 3 IU of insulin and 50 mg of adjuvant II (○) or adjuvant IV (×). The suppositories administered were prepared following formulas 1 and 2 (Table II). Each value in the figure indicates the mean and standard deviation of six rabbits.*

inside, and the other end of the segment was closed by ligation. The sac was placed into a test tube containing 40 ml of Krebs-Ringer's solution. The solution was held at 37° and aerated with an oxygen–carbon dioxide mixture (95:5). The concentration of glycerine esters in solution was determined by the assay described below.

Interaction of Glyceryl Esters with Calcium—The interaction of glyceryl esters with calcium was studied following a turbidimetric titration method previously described (3) with modifications (1).

Release of Adjuvants from Suppositories—The release of adjuvants from suppositories was studied using a previous method (4). A special apparatus[5] and a membrane filter (pore size 3.0 μm)[6] were used in the study of drug release from suppositories.

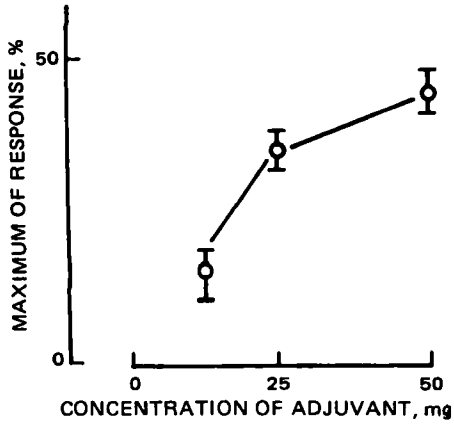

Figure 2—*Concentration effect of adjuvant II in suppositories containing 3 IU/body of insulin. The maximum response expressed in terms of the maximum change in glucose level as a percent of the initial level. Each value indicates the mean and standard deviation of six rabbits.*

[5] Toyama Sangyo Co., Ltd., Japan.
[6] Type SM, Sartorius Membranefilter GmbH, Germany.

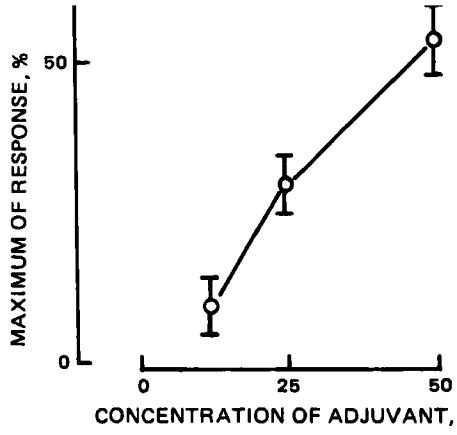

Figure 3—*Concentration effect of adjuvant IV in suppositories containing 3 IU/body of insulin. The maximum response is expressed in terms of the maximum change in glucose level as a percent of the initial level. Each value indicates the mean and standard deviation of six rabbits.*

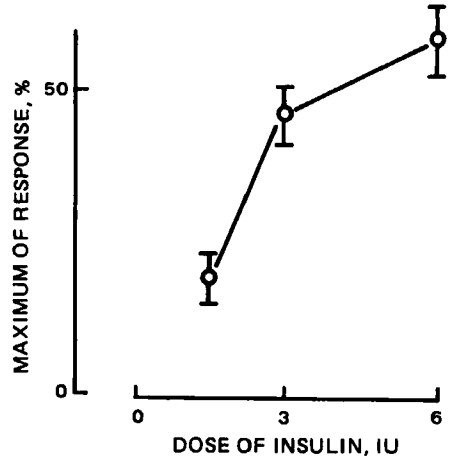

Figure 4—*Dose-response profile of insulin in rabbits administered suppositories containing 1.5–6 IU of insulin and 50 mg of adjuvant II. Response was expressed in terms of the maximum change in glucose levels as a percent of the initial level. Each value indicates the mean and standard deviation of six rabbits.*

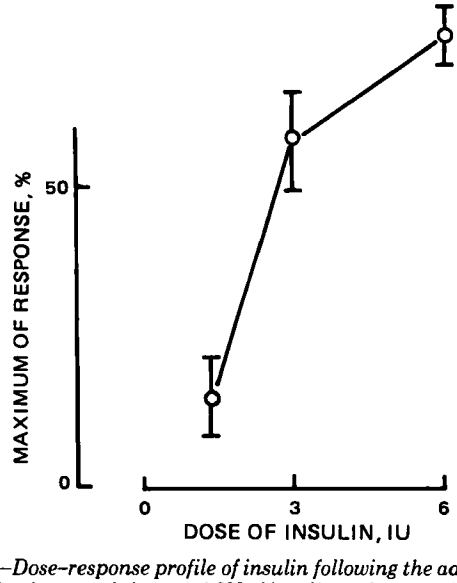

Figure 5—*Dose-response profile of insulin following the administration of suppositories containing 1.5–6 IU of insulin and 50 mg of the adjuvant IV. Response was expressed in terms of the maximum change in glucose level as a percent of the initial level. Each value indicates the mean and standard deviation of six rabbits.*

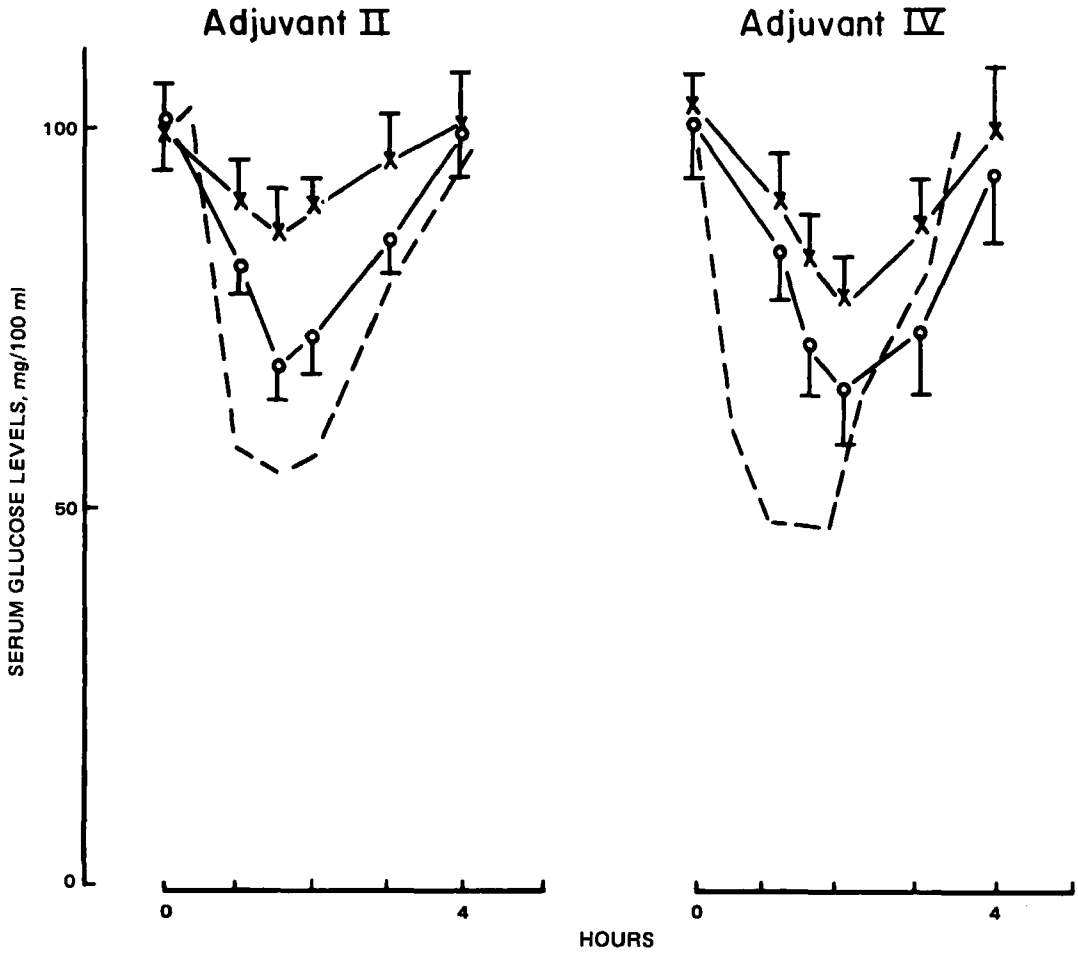

Figure 6—*Effect of calcium (O) and magnesium (X) on the promotive efficacy of adjuvants II or IV. Suppositories made according to formulas 3 and 4 (Table II) were administered at 1.0-g doses. The broken line indicates the change in serum glucose levels following the administration of an insulin suppository without calcium and magnesium. Each value indicates the mean and standard deviation of six rabbits.*

Assays—The serum glucose level was determined with the *o*-toluidine boric acid method (5) with a modification using a glucose test kit[7]. Serum insulin levels of rats were determined by a radioimmunoassay method described previously (5) using an insulin assay kit containing an agarose-bound antibody[8]. Inulin was determined as previously described (6) with the following slight modifications. A 0.5-ml sample was mixed with 1 ml of 0.1% resorcinol in 95% ethanol and 2.5 ml of 30% hydrochloric acid in a glass-stoppered test tube. The test tube was kept in a water bath at a temperature of 80 ± 0.5° for 25 min. After cooling with running water for 3 min, the color of the solution was spectrophotometrically determined at 490 nm. The concentration of glyceryl esters of acetoacetic acid in solution was determined according to a previous method (1) as a function of acetoacetate.

RESULTS AND DISCUSSION

As control experiments, 1.0-g suppositories containing glyceryl-1-monoacetoacetate (adjuvant I), glycerine-1,3-diacetoacetate (adjuvant II), glyceryl-1,2,3-triacetoacetate (adjuvant III), or 1,2-isopropylidene glyceryl-3-acetoacetate (adjuvant IV) at doses of 100 mg were administered to rabbits (Tables I and III). None of these control suppositories had any effect on the rabbits' serum glucose levels.

After rectal administration of suppositories (formulas 1 and 2 in Table II) containing 3 IU of insulin and only 50 mg of adjuvant, the serum glucose and serum insulin profiles were measured (Fig. 1). The administration of the suppositories containing adjuvants II and IV caused a dramatic decrease in glucose levels with minimums at 60–120 min and 60–90 min, respectively (Table I). Concomitantly, the serum insulin concentrations increased rapidly and reached maximum levels at 60 min for the suppositories containing adjuvant II and 30 min for adjuvant IV.

[7] Wako Pure Chemical Ind. Co., Japan.
[8] Pharmacia Co., Ltd., Japan.

Adjuvants I and III failed to reduce the serum glucose and did not increase the serum insulin level in these experiments. Adjuvants I and III also failed to enhance the rectal absorption of inulin. When 1.0-g suppositories (formula 5 in Table II) containing 25 mg of inulin/g and 50 mg of the adjuvant were rectally administered to rabbits, only adjuvants II and IV promoted inulin absorption (Table IV).

To study the effect of adjuvant concentration on the serum glucose

Table IV—Recovery of Inulin in Urine After Administration of Suppositories Containing 25 mg of Inulin/g and 50 mg of the Adjuvant at a Dose of 1.0 g of Suppository [a]

Adjuvant	Recovery of Inulin in Urine, % (4.0 hr)[b]
I	—
II	28.5 ± 1.8
III	—
IV	38.7 ± 2.1

[a] See formula 5 in Table II for suppository content. [b] Each value in the table indicates the mean ± standard deviation of six rabbits.

Table V—The Effect of Calcium and Magnesium on the Recovery of Inulin in Urine After Administration of 1.0-g Suppositories [a]

Adjuvant	Recovery of Inulin in Urine, % (4.0 hr)	
	Calcium[b]	Magnesium[c]
II	11.6 ± 2.1	6.3 ± 2.4
IV	19.8 ± 4.2	10.9 ± 3.8

[a] Each value in the table indicates the mean ± standard deviation of six rabbits. [b] See formula 6 in Table II for suppository content. [c] See formula 7 in Table II for suppository content.

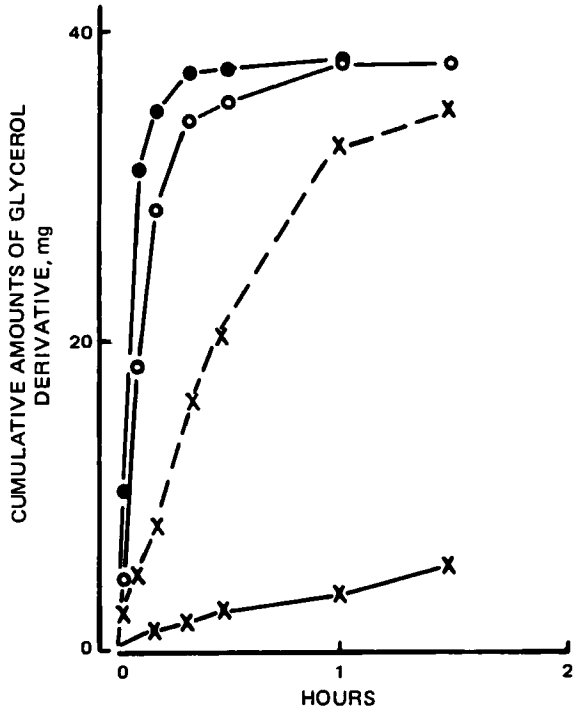

Figure 7—*Release of adjuvants I (●), II (○), III (— × —), and IV (-- × --) from triglyceride base into a saline solution.*

level, 1.0-g suppositories were prepared with concentrations ranging between 12.5 and 50 mg of adjuvant II or IV and containing 3 IU of insulin (Figs. 2 and 3). The insulin response was expressed as the maximum change in glucose relative to the initial level. With increasing adjuvant concentration in the suppositories, the maximum change in the serum glucose level increased accordingly. Rectal administration of 3 IU of insulin in the presence of 50 mg of adjuvant II or IV caused a 50% reduction in rabbit serum glucose concentration.

To study the dose–reponse profiles of insulin, 1.0-g suppositories containing 1.5–6.0 IU of insulin and 100 mg of adjuvant II were administered to rabbits (Fig. 4). The maximum response increased asymptotically with an increase in the insulin dose. Normal glucose level recovery took longer with higher concentrations of insulin. Similar results were obtained with adjuvant IV (Fig. 5).

In a previous paper (1), it was suggested that the absorption-promoting efficacy of enamine derivative was linearly related to their interacting ability with calcium ions located in the rectal membrane. In the present experiment, the addition of calcium gluconate or magnesium chloride

Table VI—Interacting Ability of Glycerol Derivatives with Calcium at pH 10.0 [a]

Adjuvant	Calcium gram per mole of compound
I	0.26
II	0.34
III	0.36
IV	0.28

[a] Gram of calcium ion interacting with 1 mole of glycerol derivative; results represent the mean of 3 determinations.

to suppositories markedly suppressed the promotive efficacy of adjuvants II and IV on the rectal absorption of insulin and inulin (Fig. 6 and Table V). The depressive effects of calcium and magnesium on the action of adjuvants were dramatically observed at doses shown in formulas 3, 4, 6, and 7 (Table II). Half doses of calcium and magnesium caused only small changes in the serum glucose compared with doses without the ions. These results appear to indicate that the promotive efficacy of glyceryl esters as well as enamine derivatives may be partly dependent on the interaction of the adjuvant with calcium and magnesium ions located in the rectal membrane. This interaction may cause transient gaps to form in the membrane allowing the insulin or inulin to permeate the rectal membrane more readily.

Even though the calcium binding ability of the four glyceryl esters was found to be similar to that of the enamine derivatives (Table VI), adjuvants I and III did not promote the rectal absorption of either insulin or inulin. Therefore, the promotive efficacy of adjuvants II and IV can not totally be explained by their ability to interact with calcium. To further explore possible absorption-enhancing mechanisms of these adjuvants, release of adjuvants from triglyceride base to water (maintained at 37° and continually stirred with a magnetic stirring bar) was studied (Fig. 7). The rates of release of the adjuvants were found to be in the order of I ($k_1 = 0.207$ min^{-1}), II ($k_1 = 0.115$ min^{-1}), IV ($k_1 = 0.029$ min^{-1}), and III (unmeasurable) as calculated by the following equation:

$$\frac{d(A_t/V)}{d_t} = k_1\left(\frac{A_0 - A_t}{V}\right) \qquad \text{(Eq. 1)}$$

where A_0 is the total amount of glycerol derivative in the system, A_t is the released amount of glycerol derivative at time t, V is the volume of the solution, and k_1 is the release rate constant.

Adjuvant III was inadequately released from the base owing to its poor water solubility. Thus, the deficiency in the promotive activity of adjuvant III may be explained by its inadequate partitioning property.

To investigate the deficiency in the promotive activity of adjuvant I, permeation of adjuvants and inulin through the excised sac of the rat rectum was studied (Fig. 8). Permeation rates were calculated using Eq. 2:

$$\frac{d(A_t/V)}{d_t} = k_2\left(\frac{A_0 - A_t}{V}\right) \qquad \text{(Eq. 2)}$$

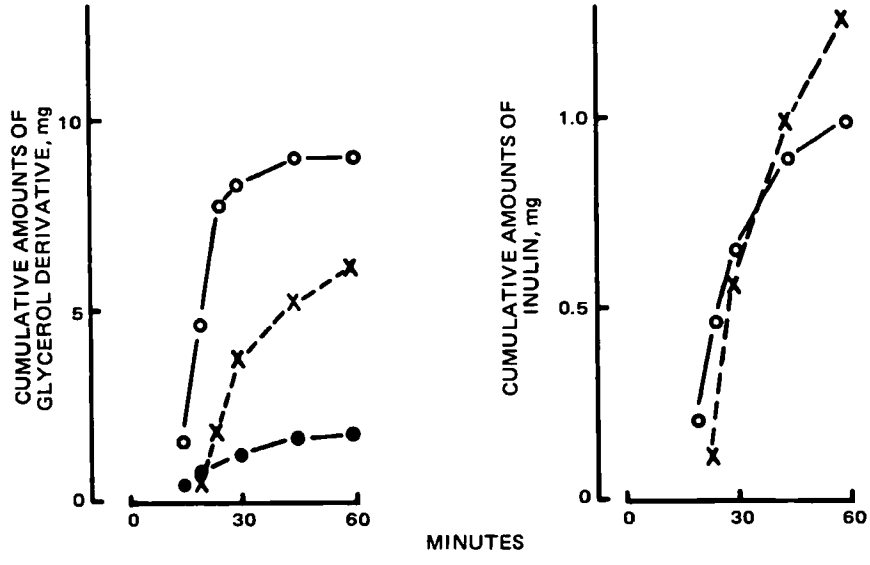

Figure 8—*Permeation of adjuvants I (●), II (○), and IV (×) with inulin through the excised sac of the rat rectum. Liquified suppositories (0.1 g) containing 10% of one of the glycerol derivatives and 30 mg of inulin/g of suppository were used.*

where all symbols are the same as before and k_2 is the permeation rate constant. Adjuvant I ($k_2 = 0.42 \times 10^2$ min^{-1}) did not readily cross the rectal membrane, possibly due to its strong lipophobicity. Adjuvants II ($k_2 = 7.29 \times 10^2$ min^{-1}) and IV ($k_2 = 1.91 \times 10^2$ min^{-1}) which are more lipophilic, easily permeated the rectal membrane and promoted the absorption of inulin.

Thus, adjuvant enhancement of rectal absorption of insulin and inulin appears to depend on at least three factors: adjuvants must be effectively released from the suppository, be able to permeate the membrane, and be able to interact with the calcium and magnesium ions in the membrane.

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Analysis and Prediction of Partition Coefficients of *meta*- and *para*-Disubstituted Benzenes in Terms of Substituent Effects

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Received November 13, 1981, from the *Department of Agricultural Chemistry, Kyoto University, Kyoto, Japan 606.* Accepted for publication May 5, 1982.

Abstract □ The hydrophobic substituent parameter for a system of *meta*- and *para*-disubstituted benzenes, XC_6H_4Y, defined as $\pi_{X/PhY} = \log P_{XC_6H_4Y} - \log P_{C_6H_5Y}$, where P is the octanol–water partition coefficient and X and Y are variable and fixed substituents, respectively, varies from one system to another, according to the variation in substituent effects on the hydrogen bonding association of substituents with solvents. Using parameters from monosubstituted benzenes, $\pi_{X/PhH}$ as the reference, the π_X values were analyzed by such relations as $\pi_{X/PhY} = a\pi_{X/PhH} + \rho_Y\sigma_X + \rho_X\sigma_Y$, where ρ_Y and ρ_X are susceptibilities of the relative hydrogen bonding association of substituents Y and X with two partitioning solvents to the electronic effect of X and Y, respectively. For substituents incapable of hydrogen bonding such as alkyl and halogen, the ρ value is 0. The parameter a is a constant $\simeq 1$. The relationship was applied in calculating log P values of disubstituted benzenes.

Keyphrases □ Partition coefficient—octanol–water, analysis and prediction, *meta*- and *para*-disubstituted benzenes in terms of substituent effects □ Disubstituted benzenes—*meta*- and *para*-, analysis and prediction of partition coefficient, substituent effects □ Structure–activity relationships—analysis and prediction of partition coefficient of *meta*- and *para*-disubstituted benzenes in terms of substituent effects

In recent years, log P values (P is the 1-octanol–water partition coefficient) have been widely used as a parameter of the hydrophobic property of organic compounds in structure–activity studies (1). Log P values of complex molecules often can be calculated from those of suitable reference molecules and π values, where π is defined as $\pi_X = \log P_X - \log P_H$ (P_X is the coefficient value of a derivative on which the substituent X is carried and P_H is the coefficient value of a reference).

As pointed out earlier, however, the π value varies from one solute system to another (2). It was suggested that the variation in π values of aromatic substituents in various disubstituted benzene systems should be rationalized in terms of electronic interactions between substituents when no significant steric interaction is involved (2). For *meta*- and *para*-X substituents in disubstituted benzene systems of the type XC_6H_4Y, it was proposed that the variation in π relative to the value obtained for the monosubstituted benzene system, XC_6H_4H, depends on electronic interaction between each of the X-substituents and the fixed function Y, and is formulated, in general, as:

$$\Delta\pi = \pi_{X/PhY} - \pi_{X/PhH} = \rho_Y\sigma_X + \rho_X\sigma_Y \quad \text{(Eq. 1)}$$

where ρ_Y and ρ_X are the susceptibility constants of substituents Y and X to the solubility-modifying effects of substituents X and Y, respectively.

Since interest in the use of log P values in quantitative structure–activity studies is growing rapidly, it is important to clarify the composition of π values from various solute systems. The present report examines how far relations such as Eq. 1 can be applied in predicting π values for calculation of log P values.

EXPERIMENTAL

Solute Systems—Seventeen sets of π values (a total of 360 values) were used for the study. They were calculated from log P values of 17 disubstituted benzene solute systems and the corresponding reference monosubstituted benzenes. The majority of the log P values were taken from earlier reports (2, 3).

Several were determined[1] according to the reported procedure (2). Other values, *e.g.*, those for substituted benzamides (4), formanilides (5), acetanilides (6), and pyridines (7), are from the literature.

General Procedure—It was assumed that the solubility-modifying effect of substituent X on Y, as well as that of Y on X, was due primarily to the variation in hydrogen bonding association of substituents with solvents, according to the variation in the electronic environment of substituents X and Y. In actual examination of the applicability of Eq. 1, analysis was performed according to equations where $\pi_{X/PhY}$ and $\pi_{X/PhH}$ were used as dependent and independent variables, respectively. Although it should be close to 1, the slope of the $\pi_{X/PhH}$ term is not necessarily equal to 1. To avoid giving the unsubstituted solute excessive weight, an intercept term, c, has been included which should be close to 0. Equation 2 is employed when the fixed substituent Y is capable of hydrogen bonding:

$$\pi_{X/PhY} = a\pi_{X/PhH} + \rho_Y\sigma_X + \rho_X\sigma_Y(meta) + \rho_X\sigma_Y(para) + c \quad \text{(Eq. 2)}$$

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