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161. Kiichiro Kakemi, Hitoshi Sezaki, Shikifumi Kitazawa, and Katsuhiko Okumura*1 : Studies on the Pharmaceutical Potentiation of Drugs. Ⅳ.*2 Biopharmaceutical Study on the Derivatives of *p*-Aminosalicylic Acid.

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Stabilities, absorption and excretion profiles, and biotransformations of the ω-substituted alkyl-PAS were investigated. The observations presented here support the conclusion that derivatives are stable in gastro-intestinal tract and absorbed more readily than that parent compound. Derivatives with long alkyl chain have characteristics of sustained release and can maintain high level in body fluid for long period. These derivatives are metabolized to the same extent regardless of their differences of the structure.

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The wider spread use of *p*-aminosalicylic acid (PAS) or its sodium or calcium salt as a chemotherapeutic agent in the treatment of tuberculosis has directed much effort towards rendering to diminish the large dosage of this drug. A wide variety of pharmaceutical formulations such as powders, tablets and granules have appeared but do little to potentiate or diminish the dosage.

In the previous papers,[1~3] a number of ω-substituted alkyl *p*-aminosalicylates were synthesized to the same purpose by the chemical modifications of the drug, and it was demonstrated that properties that govern such biological inactivation processes of the parent compound as protein binding, lipid solubility and antagonistic effect by *p*-aminobenzoic acid (PABA) were greatly improved. It was also found that these characteristics were highly influenced by the nature of both of chemical constitution of substituents on the end of and the length of the alkyl chain of the derivatives.

The present study is chiefly concerned with the problems which occurred in the course of practical usage of these derivatives, that is, biopharmaceutical characteristics such as stabilities in the gastro-intestinal tract, absorption and excretion profiles and their extents of biotransformation are investigated.

Enzymatic degradations of these derivatives were examined by measuring the residual amount in the solutions which had been recirculated for one hour through the rat small intestine or stored for one hour in the rat stomach.

Non-enzymatic degradations were measured in various buffered solutions of pH 1 to 10 under the temperature of 37° and 50°.

Quantitative determinations of the intestinal absorption of the derivatives were carried out by the method previously reported from this laboratory.[4]

The excretion of these derivatives were investigated after oral administration to the rabbit and both the amount of total amines and intact compound were determined simultaneously in one hour intervals from one to eight-hour-urine.

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*2 Part Ⅲ: This Bulletin, **15**, 925 (1967).

1) This Bulletin, in press.
2) *Ibid.*, in press.
3) *Ibid.*, **15**, 925 (1967).
4) K. Kakemi, T. Arita, and T. Koizumi : This Bulletin, **12**, 421 (1964).

Experimental

Absorption Study——Male Wister arbino rats, weighing 150 to 190 g., were fasted for 20 to 25 hr. prior to the experiments but had access to drinking water at all times. The animals were anesthetized with sodium pentobarbital by intraperitoneal injection in 4.5 to 5.0 mg. per 100 g. of the body weight. The small intestine was exposed by a midline abdominal incision, and was cannulated for in situ recirculation. The animals were maintained under anesthesia for the entire course of the experiments.

The intestine was first perfused with 50 ml. of 0.9% sodium chloride solution, previously warmed to 37°, and then with 20 ml. of the solution of the derivative examined. The tubings attached to the inflow and the outflow cannulae were then transferred to a flask contains 50 ml. of the solution. The solution was then continuously circulated through the small intestine for 1 hr. at 37°. The solution which usually contained 100 μg. per ml. of the derivative was prepared with the isotonic buffered solution. The component of the solution are listed in Table I. One ml. of the recirculated solution was pippeted out at the intervals of 15, 30 and 60 min. after zero time. The zero time was set at 10 min. after the starting of the recirculation.

Table I. The Component of isotonic buffered Solutions used in the Absorption Study

pH	Citric acid (g.)	$Na_2HPO_4 \cdot 12H_2O$ (g.)	KH_2PO_4 (g.)	NaCl (g.)	H_2O
4.0	15.3	31.7	—	—	
5.2	9.2	36.5	—	—	to make 1000 ml.
6.5	—	6.0	4.7	6.0	
7.5	—	13.2	1.3	6.0	

Extent of the absorption was calculated on the basis of the decrease of the concentration in the solution recirculated. Phenol red was used as a marker to determine the extent of volume change of the solution. Any changes in the volume of the solution during the period of the procedure would be reflected by a corresponding change in phenol red concentration.

In the case of hardly soluble derivatives, a certain amount of the substance was first dissolved in 0.5 ml. of dimethylacetamide and then diluted with the isotonic buffered solution to make 100 ml. under stirring. The final concentration of thus obtained suspension was 50 μg. per ml. These suspensions which contained the solute in the state of particles in 1～50 μ in diameters, were used for the recirculation. Effect of dimethylacetamide was checked to be negligible under the experimental condition.

Enzymatic Stability——Intestinal recirculated fluid obtained from the above experiments was adjusted to pH 3 by addition of *N*/10 HCl solution and then extracted twice with a certain amount of ethylacetate. Extracts were combined and evaporated to dryness *in vacuo* at room temperature. The residue was appropriately diluted with acetone, and then spotted for the thin-layer chromatogram. Solvents used for the developments were *n*-butanol for ω-diphenylaminopentyl-PAS and a mixture of chloroform and acetone (9:1) for ethyl-PAS. The spots were stained yellow with Ehrlich's reagent. In the cases of investigations of stability in stomach, three ml. of the solution of the derivative was stored in the rat stomach for one hour and then treated similarly as mentioned above.

Non-enzymatic Stability——Two ml. of the solution containing 50 μg./ml. of a derivative in buffered solution was placed in a glass-stoppered test tube. The pH of the solution was buffered from 1 to 10 respectively. The component of the buffered solution of each pH are shown in Table II. After being kept for 2 hr. at 37° or 50°, the extent of degradation was measured spectrophotometrically.

Table II. The Component of buffered Solutions used in the Studies of non-enzymatic Stability of the Derivatives

pH	Citric acid (g.)	$Na_2HPO_4 \cdot 12H_2O$ (g.)	KH_2PO_4 (g.)	NaOH (g.)	H_3BO_3 (g.)	KCl (g.)	Na_2CO_3 (g.)	$NaHCO_3$ (g.)	H_2O
1.0				0.1*N* HCl					
3.0	26.6	23.1	—	—	—	—	—	—	to make 1000 ml.
5.0	10.1	35.9	—	—	—	—	—	—	
7.0	—	14.3	3.6	—	—	—	—	—	
8.0	—	22.7	0.5	—	—	—	—	—	
9.0	—	—	—	0.9	3.1	3.7	—	—	
10.0	—	—	—	—	—	—	12.7	6.8	

Analytical Method——Compounds were extracted with chloroform after adjusting of pH of the solution to 10 by adding Mentzel buffer and then the chloroform layers were determined spectrophotometrically at the wave length of 303 mμ to 310 mμ using Shimadzu spectrophotometer type QV-50. PAS was determined by the method followed by Charney.[5]

Urinary Excretion Study——Male albino rabbit, weighing 2.9 to 3.1 kg., were used in the study. Animals were fastened and the substances were administered orally through stomach cannulaes. Dose of these administrations were 500 mg. per 3 kg. of the body weight. In all cases compounds were triturated physical size of not more than 48-meshes and suspended in 70 ml. of water. By this method, it was possible to minimize any error arising from the dissolution of the substances in the gastro-intestinal tract. Urinary excretion tests were conducted by collecting urine one hour interval up to eight hours after ingestion of the doses. For the determination of total amines in urine, analytical method by Pesez[6] was followed. Inact substances excreted were assayed by the method decribed above.

Results and Discussion

Stability

No significant non-enzymatic breakdown was observed in the region of biological pH of 1 to 8 and at the temperature of 37°, but the degradation was promoted with increasing pH to 9 and 10, and raising the temperature up to 50°. The results of the investigation on enzymatic degradation are shown in Fig. 1. A faint spot observed in B and C of the thin-layer chromatogram was located as PAS. These derivatives are sufficiently stable in the stomach.

Since faint spots observed in B and C of the thin-layer chromatogram were located as PAS, hydrolysis to PAS itself was proceeded in some extent by enzymes located in the small intestine. But comparing to the amount of intact substance, extent of the hydrolysis was regraded to be negligible. In view of these facts, it might be concluded that these derivatives are more stable in the gastro-intestinal tract than the parent compound. Considering the Heller's observations[7] that 1~4% of PAS are decarboxylated in the acidity of the stomach, esterification of the compound seems favorable from the point of drug stability.

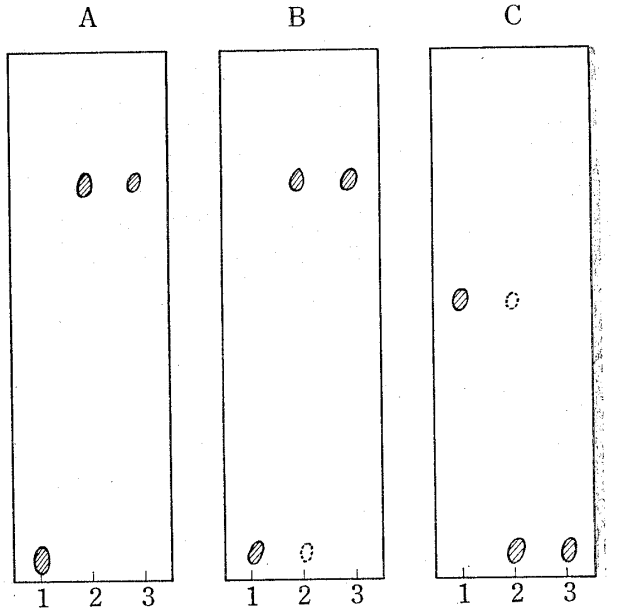

Fig. 1. Stability of the Derivatives which was stored in the Stomach and recirculated in G-I Tract for One Hour and then submitted to Thin-layer-chromatography

Absorption of Soluble PAS Derivatives

In previous reports, partition coefficients of PAS derivatives between heptane and buffered solution having pH 7.4 were investigated and it was observed that these derivatives were more oil soluble than the parent compound. On the basis of lipoid

5) J. Charney and M. Kuma: Am. Rev. Tuberc., **64**, 577 (1951).
6) M. Pesez: Bull. Soc. Chim. Biol., **31**, 1369 (1949).
7) A. Heller, *et al.*: Am. Rev. Tub., **75**, 71 (1957).

theory,[8] this characteristics seems to assure the rapid absorption of these derivatives through the gastro-intestinal tract. Table Ⅲ shows the partition coefficients and the results of the absorption study of these derivatives at pH 6.5. Most of the derivatives are rapidly absorbed from the rat small intestine at pH 6.5, biological pH of the gut.

Table Ⅲ. Absorption Rates of Soluble PAS Derivatives (pH 6.5)

Compounds	Partition coefficients	Absorption rates % absorbed in one hour (%)
PAS	0.09	23.3
Ethyl-PAS	9.24	90.4
Hexyl-PAS	25.3	91.2
Chloroethyl-PAS	3.1	78.3
Chlorodecyl-PAS	48.1	81.7
Hydroxyethyl-PAS	0.15	81.9
Diphenylaminoethyl-PAS	17.6	36.3
Diphenylaminopentyl-PAS	36.2	33.1
Phenylethylaminoethyl-PAS	6.3	90.3

These data are the mean of three experiments.

It is suggested from the table that the effect of the ω-substituents are dominant while the length of the alkyl chain, which is closely related to the lipid solubility, slightly influences the absorption characteristics. It is considered that for compounds such as these derivatives which are too lipid soluble, the rate of absorption are no more limited by their oil water partition coefficients.

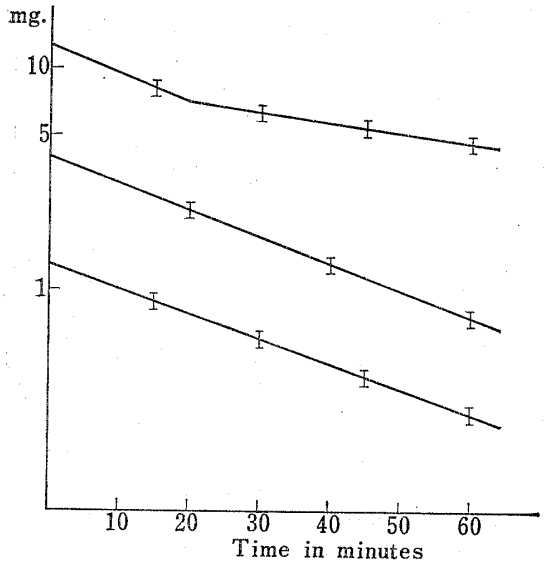

Fig. 2. Logarithmic Plot of Drug Remaining in Recirculating Solution of ω-Hydroxyethyl-PAS at pH 6.5

Effect of solute concentration on the intestinal absorption was investigated with ω-hydroxyethyl derivatives using initial concentration of 30 μg./ml., 100 μg./ml. and 300 μg./ml. As shown in Fig. 2, three straight lines with the same slope were obtained in the semi-logarithmic plot, which suggests that these derivatives are transferred through the intestinal membrane by simple passive transport. Slight deviation of the line corresponding to 300 μg./ml. solution suggested the possibility of a progressive increase of the reverse reaction, perhaps due to high blood concentration, which was caused by rapid absorption of the derivatives.

Fig. 3 and 4 shows the effects of pH on the absorption of the derivatives. Ethyl-PAS, ω-Chloroethyl-PAS, ω-hydroxyethyl-PAS are absorbed rapidly from the solution having various pHs investigated here. Absorption rate of chloroethyl-PAS was almost constant over wide range of pH, whereas ethyl-PAS and ω-hydroxyethyl-PAS were slightly depended upon pH of the medium. While absorption of ω-diphenylaminopentyl-PAS and ω-phenylethylaminoethyl-PAS decrease in low pH region, which may be attributed to the dissociation of tertiary amine of the substituents in such medium.

8) L. S. Schanker, P. A. Shore, B. B. Brodie, *et al.* : J. Pharmacol. Exptl. Therap., **120**, 528 (1957). *Idem*: *Ibid.*, **123**, 81 (1958).

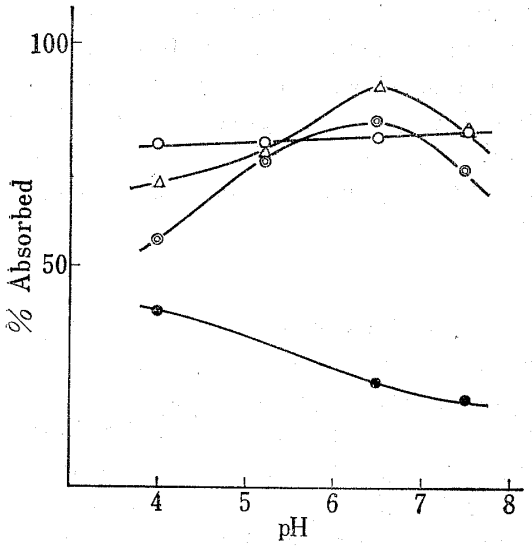

Fig. 3. Absorption Rate of PAS Derivatives through the Rat Intestine from the Solution of various pH (1 hr.)

● PAS
○ chloroethyl-PAS
△ ethyl-PAS
◎ hydroxyethyl-PAS

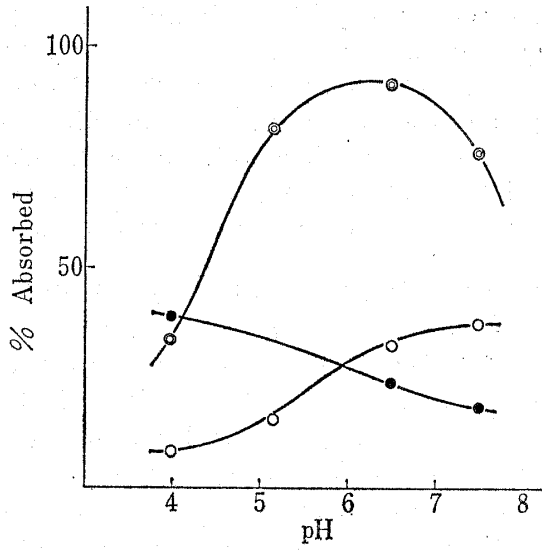

Fig. 4. Absorption Rate of PAS Derivatives through the Rat Intestine from the Solution of various pH (1 hr.)

● PAS
○ diphenylaminopentyl-PAS
◎ phenylethylaminoethyl-PAS

Absorption of Less Soluble PAS Derivatives

It has been recognized during the course of the syntheses of these derivatives that, in one hand, esterification of PAS increased the lipid solubility but, on the other hand, it decreased water solubility.

Table Ⅳ indicates that despite their low intrinsic solubility these derivatives are also absorbed readily from the intestinal tract. Fig. 5 illustrated plots of the residual amount of the derivatives against time. Since these derivatives were recirculated in a state of suspensions, their apparent rates of absorption seem to follow zero-order kinetics.

Table Ⅳ. Absorption Rates of poorly soluble PAS Derivatives (pH 6.5)

Compounds	P. C.	% absorbed
Decyl-PAS	121	49.5
Hydroxydecyl-PAS	17.3	62.5
Diphenylaminodecyl-PAS	70.1	52.0
Phenylethylaminodecyl-PAS	42.3	46.3

These data are the mean of three experiments.

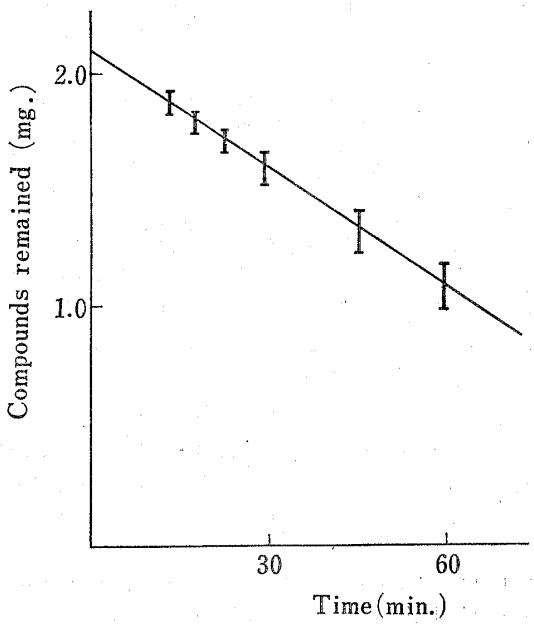

Fig. 5. The Residual Amount of Decyl-PAS *vs.* Time of Recirculation

Decreasing in water solubility often present formidable obstacles for the absorption of the drug through gastro-intestinal tract, but from pharmaceutical standpoint of view and if the rate of solution of the substances are not so slow, this sometimes endows a compound another merit, by controlling the rate of solution in gastro-intestinal tract, the nature of sustained-release form. Considering these results, it can be understood that the phsiological availabilities of these compounds are not so decreased in spite of their low intrinsic solubilities as described above.

Urinary Excretion and Biotransformation

It is well known that PAS is highly metabolized and excreted rapidly through the kidney,[9,10] but little is known about the excretion and the biotransformation of such derivatives as esters of PAS. Since Nakao[11] identified nine metabolic products of PAS from human urine after oral administration, and in the cases of the derivatives, the metabolic processes seemed to be more complicated, the amount of excreted PAS derivatives, including the inact esters, were determined as total amines by Pesez's method[8] and the latter were measured respectively by the method described above.

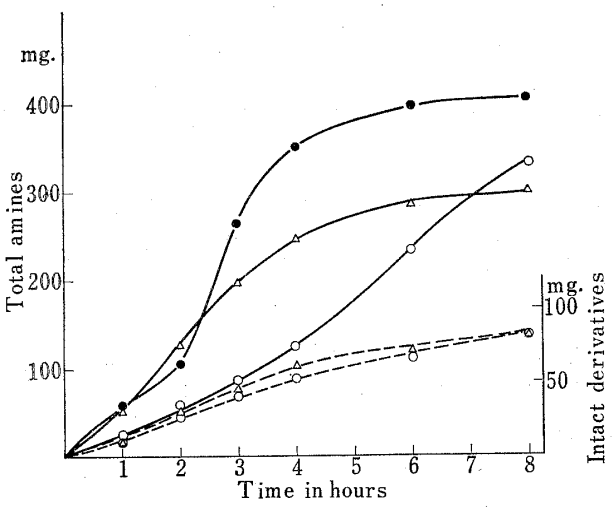

Fig. 6. Cumulative Curves of Urinary Excretion of Total Amines and of Intact Esteric Derivatives after the Administration of PAS Derivatives. Continuous

Lines present total amines and dotted lines present the intact derivatives.
● PAS ○ Decyl-PAS
△ Ethyl-PAS

Fig. 6 shows the urinary excretion curves of total amines obtained after oral administration of PAS to the rabbit. Eight hours after the ingestion of the compound, the excretion seemed to be complete. In the cases of ethyl-PAS and decyl-PAS, however, excretion was still in progress after eight hours and, at the same time, considerable amount of unchanged esters were excreted in urine, which reflects the persistence of biological active unchanged esters in the body fluid.

From this observation, attentions were turned to tracing the movements of esters by following the rates of urinary excretion. Fig. 7 and 8 are the curves of urinary excretion rates *vs.* time obtained by the method of Nelson.[12] Although this is obviously an indirect measurement of excretion rate of the absorption and elimination of the compound in the body fluid of the rabbit. The maximum amount of ethyl-PAS is attained after 30 min. of the oral administration and this confirms the rapid absorption of the water soluble esteric compound, while decyl-PAS produces a maximum after 1.5 to 2 hours of the administration and maintains for long time. In view of the fact, it is apparent that these hardly soluble derivatives can prolong their durations of action in animal body by sustained release characteristics due to their slow rate of solution. From a consideration of rapid elimination of PAS from the body fluid, esterification of PAS by long alkyl chain seems favorable from the point of prolongation of duration time in animal body.

9) D. M. Tennent, M. L. Leland: J. Biol. Chem. **177**, 873 (1949).
10) E. N. Debb, G. R. Vitagliano: J. Am. Pharm. Assoc., **44**, 182 (1955).
11) M. Nakao: J. Biochem. **44**, 327, 433, 477 (1957).
12) E. Nelson: J. Am. Pharm. Assoc., **48**, 489 (1959); **49**, 54, 437 (1960).

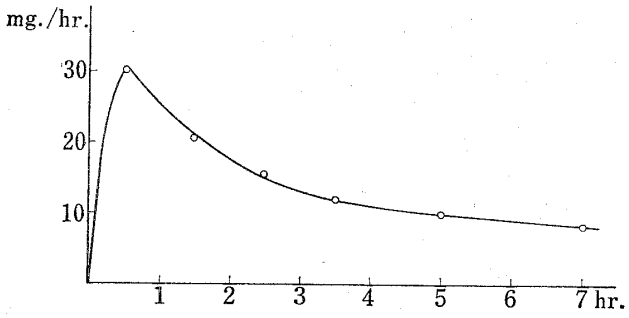

Fig. 7. Curve of Urinary Excretion Rate *vs.* Time after the Administration of ethyl-PAS to Rabbit

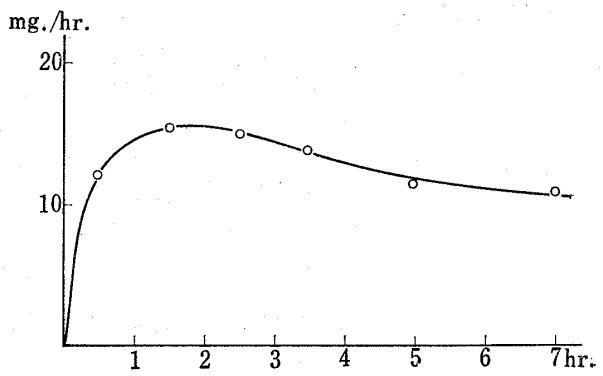

Fig. 8. Curve of Urinary Excretion Rate *vs.* Time after the Administration of Decyl-PAS to Rabbit

Ratios of the amount of unchanged esters to the amount of total amines are calculated in four and eight hours urine respectively. Results are listed in Table V. From these data, it is suggested that these derivatives are metabolized to almost the same extent regardless of their different chemical structures.

TABLE V. The Amount of unchanged esteric Derivatives which was found in Four-hour-urine and Eight-hour-urine

Compounds	in 4 hr. Urine (%)	in 8 hr. Urine (%)
PAS	18.3	14.2
Ethyl-PAS	25.5	26.0
Decyl-PAS	45.3	24.7
Hydroxyethyl-PAS	21.7	14.1
Hydroxydecyl-PAS	32.1	23.8
Chloroethyl-PAS	27.1	24.2
Chlorodecyl-PAS	39.1	26.1
Bis-ethyl	54.3	40.3
Bis-decyl	51.2	27.4
Diphenylaminoethyl-PAS	35.1	26.3
Diphenylaminodecyl-PAS	38.3	20.6
Phenylethylaminoethyl-PAS	31.2	27.4
Phenylethylaminodecyl-PAS	33.2	23.5

These data are the mean of three experiments.

The differences in 4 hours urine might be caused from the difference of staying period in body fluid, that is, absorption of the derivatives that have long alkyl chain were delayed due to their low rate of solution and their staying periods in body fluid were seemed to be short to be metabolized, while the cases of water soluble derivatives, they are readily dissolved and rapidly absorbed and can stay in body fluid for longer time to be metabolized, but these differences are eliminated in eight hours urine. Comparing the extent of these derivatives to that of PAS, it is clear that these compounds are less metabolized in body fluid than that of PAS.

Data obtained in this study support the conclusions that these derivatives are stable, more easily absorbed from the gastro-intestinal tract, and less metabolized than the parent compound, and that esterification of PAS by long alkyl chain might be favored from the point of prolongation of duration time in animal body.