83. **Hisao Tsukamoto and Akira Yamamoto**: Metabolism of Drugs. IV.* The Metabolic Fate of *p*-Aminosalicylic Acid in the Rabbit. (1).

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p-Aminosalicylic acid (PAS) has been widely used in the treatment of tuberculosis. There are consequently extensive literatures on the metabolism and excretion of this drug.

Venkataraman and others[1] reported that the urine of rabbits administered PAS contained N-acetylated *p*-aminosalicylic acid (Ac-PAS) and unchanged PAS, but increasing amounts of glucuronic acid and ethereal sulfate in the urine were not observed. Furthermore the PAS-glycine conjugate (*p*-aminosalicyluric acid) and PAS-oxidation product were not detected. Several workers[2~5] reported the occurrence of the above metabolites in the human.

In spite of these above-mentioned works, the metabolic products of PAS still remain to be clarified and further informations are required as to the biological behavior of the compound in rabbits.

The present investigation is a comparative study of the matabolism of PAS and Ac-PAS, which is the main metabolic product of PAS. It has been indicated that the glucuronic acid conjugation of Ac-PAS is much less than that of PAS.

Meterials and Methods

The sodium salt of PAS was used. Ac-PAS was synthesized by the method described by Romeo.[6] In all properties this synthetic compound agreed with Ac-PAS excreted in the urine of rabbits administered PAS.

The animals used were male rabbits weighing 2.5~3.3 kg. They were housed in metabolism cages and fed 'Okara' (soybean curd residue) only. Either PAS (1 g./kg. body wt.) or Ac-PAS (1.27 g./kg. body wt.), suspended in simple syrup, was administered by stomach tube. Urinary collections were made every 24 hrs. after the administration of the drugs. The decomposition of metabolites was prevented by the addition of toluene to the collection bottles.

PAS was determined spectrophotometrically by using Ehrlich's reagent for aromatic amines and iron reagent for phenols, which was described by Venkataraman.[1] Free amines were estimated by adding 5 cc. of 8% *p*-toluenesulfonic acid (TSA) to 5 cc. of a suitably diluted urine sample, followed by 1 cc. of Ehrlich's reagent (*p*-dimethylaminobenzaldehyde). The color was read at 440 mμ with a similarly treated normal urine blank for 100% transmission. For total amines a similarly treated urine was hydrolyzed in a boiling water bath for 1 hr. The tube was then cooled and 1 cc. of the Ehrlich's reagent was added. Under the above conditions, PAS and Ac-PAS were completely decarboxylated to *m*-aminophenol (MAP). The results calculated as MAP were converted to PAS or Ac-PAS by multiplying by the factor 153/109 or 195/109, respectively.

For the iron reaction, 5 cc. of 8% TSA was added to 5 cc. of urine so diluted as to contain 0.2~1.2 mg. of PAS, followed by 2 cc. of the iron reagent ($Fe(NO_3)_3 \cdot 9H_2O$), and the pink color was read at 500 mμ with a normal urine blank for 100% transmission.

Glucuronic acid was estimated by the modified naphthoresorcinol method described by Hanson, Mills, and Williams.[7] To 2 cc. of the urine, diluted so as to contain 10~80 μg./cc. of glucuronic acid, 2 cc. of 0.25% naphthoresorcinol solution and conc. HCl were added. The mixture was heated in a boiling water bath for 2 hrs., cooled in ice water for 10 mins., and the solution was

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extracted twice with 5 cc. of isoamyl alcohol. To the alcohol layer, 1 cc. of abs. EtOH was added and the blue violet color developed was read at 610 mμ.

Ethereal sulfate excretion was obtained from the difference between the inorganic sulfate and total sulfate excretions and these values were determined by means of the benzidine precipitation procedure.[8] PAS did not interfere with the estimations of glucuronic acid and ethereal sulfate. All the foregoing color estimations were carried out with a Hitachi photoelectric absorptiometer.

The paper chromatography was carried out using a Toyo Roshi No. 50 with 0.01 cc. of the urine. The chromatogram was developed with a mixture of BuOH:AcOH:H_2O(40:10:50) by the ascending method for 17 hrs. The paper sections containing the metabolites were detected by the fluoresecence under an ultraviolet light, Ehrlich's reagent, iron reagent, diazo reagent (Tsuda reagent, 1-(diethylaminoethylamino)naphthalene)), and aniline phthalate[9] for glucuronic acid.

Results

Excretion of Free Amine, Conjugated Amine and Free Phenol after the Administrations of PAS and Ac-PAS:

In Table I, the results of urinary excretion of PAS, based on the Ehrlich and iron reactions, are summarized. Free amine excreted was 23~60% of the dose and conjugated amines was 30~52% of the total amount of PAS excreted. The values of total excreted PAS obtained by the Ehrlich reaction showed relatively close values in comparison with that by the iron reaction. This suggests that PAS does not undergo a recognizable decarboxylation *in vivo* because MAP is negative to the iron reaction. The optical density observed in the iron reaction with experimental urine is due to the presence of the free and acetylated PAS. These two compounds showed a ratio of 1.5 to 1 in molar equivalents in the color intensity and therefore, the values for PAS and acetylated PAS obtained by the Ehrlich reaction with experimental urine were used to calculate the expected individual optical density in the iron reaction.

Table I. Excretion of Free Amine, Conjugated Amine, and Free Phenol in the Urine of Rabbits after Oral Administration of PAS (Each dose: PAS 1 g./kg. body wt.)

Rabbit No.	Dose g.	By Ehrlich reaction: Free amine %		Total PAS %		Conjugated amine %		By iron reaction: Free amine %	Conjugated amine %
		24 hrs.	48 hrs.	24 hrs.	48 hrs.	24 hrs.	48 hrs.	48 hrs.	48 hrs.
1	3.12	22.1	23.6	38.5	40.4	16.3	16.8	26.1	17.4
2	3.03	53.4	55.5	92.1	95.7	38.7	40.2	51.2	41.0
3	3.15	44.2	45.6	63.5	67.1	18.3	21.5	52.9	27.3
4	2.56	34.0	38.8	72.7	81.3	38.7	42.5	40.2	41.6
5	2.58	54.8	60.0	79.2	86.2	24.4	26.2	59.4	26.8

After the administration of Ac-PAS the excretion was very sluggish in comparison with that of PAS, as indicated in Table II. About 42~68% of the dose was excreted unchanged in the urine and about 1% of the dose was free amino compound. Free amines were not detected in

Table II. Excretion of Free Amine, Conjugated Amine, and Free Phenol in the Urine of Rabbits after Oral Administration of Ac-PAS (Each dose: 1.27 g./kg. body wt.)

Rabbit No.	Dose g.	By Ehrlich reaction: Free amine %		Total Ac-PAS %			By iron reaction: Free amine %	Conjugated amine %
		24 hrs.	48 hrs.	24 hrs.	48 hrs.	72 hrs.	48 hrs.	72 hrs.
1	3.22	0.35	0.76	22.2	40.9	42.3	0.82	41.0
2	3.23	0.44	0.93	29.1	51.0	51.5	0.98	50.3
3	3.23	0.63	1.28	52.9	68.2	68.5	0.99	67.1
4	2.88	0.23	0.53	24.8	50.9	54.2	0.67	55.9
7	2.94	0.35	0.74	19.1	66.8	67.2	0.72	69.3

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urine excreted 48 hrs. later. These results are summarized in Table II. It appears Ac-PAS also does not undergo a recognizable decarboxylation for reasons previously stated.

Excretion of Glucuronic Acid in the Urine of Rabbits after the Administration of PAS:

In the case of rabbits, the range of glucuronic acid output was 50~150 mg. per day in the normal urine. Therefore, the increase of glucuronic acid output was calculated on the basis of average values of the normal urine.

As indicated in Table III, in the 24 hr. period after the administration of PAS, which is the period of maximum excretion of PAS, the glucuronic acid output apparently increased. The average increase in 5 rabbits was 121 mg. from a dose of 1 g./kg. body wt. and PAS conjugated with glucuronic acid was equivalent to 3.3% of the dose.

In earlier experiments,[1] in which the dosage level was below 0.15 g./kg., the increase of glucuronic acid output was not indicated and the reason may be that the increase is so small that it is included in the experimental errors. It is, therefere, believed that if greater dosages of PAS were administered, its glucuronic acid conjugation may be temporarily substituted for acetylation.

Table III. Excretion of Glucuronic Acid in the Urine of Rabbits after Oral Administration of PAS (Administered on the 2nd day)

Rabbit No.	Dose g./kg.	Dose g.	Excreted glucuronic acid, mg./day day 1	2	3	4	5
2	1.00	2.94	151	86	253	95	
3	1.00	3.10	115	120	230	71	
4	1.00	2.50	73	74	180	103	
5	1.00	2.50	56	80	201	81	
7	0.25	0.52	80	104	126	139	65
4	0.50	1.22	75	65	135	133	53
1	1.00	3.10	56	90	176	69	112
1	1.50	4.32	98	90	203	90	78
2	2.00	5.52	108	111	284	127	120
3	2.50	7.50	126	132	355	87	76

In order to investigate this matter, doses of PAS varying from 0.25~2.5 g./kg., were administered. The results are summarized in Table IV and Fig. 1. The glucuronic acid output increased by degrees in proportion to the increase of dosage. Fig.1 shows that the percentage of PAS conjugated with glucuronic acid decreases rapidly until a dosage level of 1 g./kg. is reached, but above the level of 1 g./kg., the percentage conjugated with glucuronic acid remains fairly constant.

Table IV. Variation in the Percentage of PAS Conjugated with Glucuronic Acid with Different Dosages

Rabbit No.	Dose g./kg.	Dose g.	Extra glucuronic acid mg.	Conjugated PAS mg.	Conjugated PAS %
7	0.25	0.52	37	29	5.8
4	0.50	1.22	54	42	3.5
1	1.00	3.10	105	82	2.3
1	1.50	4.35	122	96	2.2
2	2.00	5.52	159	125	2.2
3	2.50	7.50	251	198	2.6

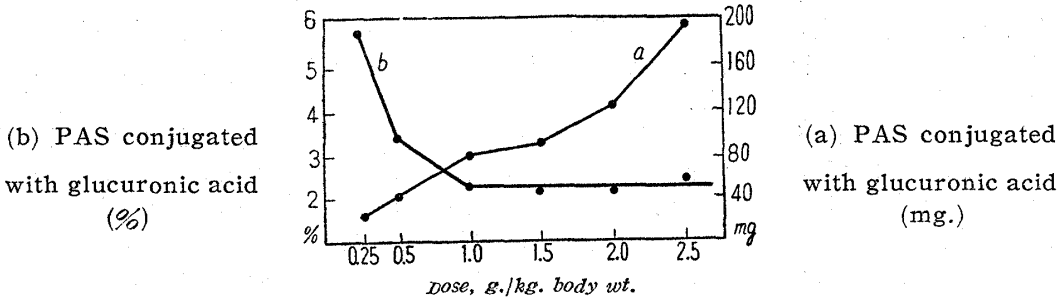

Fig. 1. Variation in the Percentage of PAS Conjugated with Glucuronic Acid with Different Dosages

Urinary Excretion of Glucuronic Acid after the administration of Ac-PAS:

On the second day, the period of maximum excretion of Ac-PAS, the increase of glucuronic acid output was nil or very small. These values were not indicative of significant conjugation in comparison with that of PAS, even at a dosage level of 2.54 g./kg. The results are summarized in Table V.

Table V. Excretion of Glucuronic Acid in the Urine of Rabbits after Oral Administration of Ac-PAS (Administered on the 2nd day)

Rabbit No.	Dose		Excreted glucuronic acid, mg./day						
	g./kg.	g.	Day 1	2	3	4	5	6	7
1	1.27	3.70	64	84	70	65	79	112	58
2	1.27	3.34	140	112	85	153	145	112	117
3	1.27	3.53	75	91	68	99	89	104	86
4	1.27	3.01	99	99	94	131	108	128	79
7	1.27	2.64	61	87	68	52	151	110	90
4	2.54	5.84	79	99	71	86	120	120	111

Paper Chromatography of the Urine of Rabbits administered PAS and Ac-PAS:

When the urine of rabbits administered PAS was developed on a filter paper for 17 hrs., the chromatogram indicated 6 yellow spots (Rf 0.8, 0.54, 0.4, 0.28, 0.24, and 0.2), on spraying the Ehrlich's reagent. The spot at Rf 0.4 was identified as a normal substance by comparison with normal urine and Rf 0.8 was that of PAS by comparison with authentic PAS. These spots were colored blue violet by the diazo reagent. The spot at Rf 0.83 was fluorescent under ultraviolet light. This value was identical with the Rf value of authentic Ac-PAS. The spots at Rf 0.83 were connected and colored purple with iron reagent. This was a mixture of PAS and Ac-PAS.

The section of Rf 0.28 was eluted with hot water and showed a strong naphthoresorcinol reaction for glucuronic acid. It is considered that this might be the PAS-glucuronide. This substance reacted with the above three coloration reagents, but only weakly with the iron reagent.

On a large filter paper (40×40 cm.), 0.5 cc. of urine was developed and the section of Rf 0.28, which was previously tested by color reagents, was cut off, extracted twice with 5 cc. portions of hot water, and filtered. The filtrate was concentrated to 0.2 cc. under reduced pressure. This solution, when developed on a filter paper, did not show a brown spot with aniline phthalate for glucuronic acid (Rf 0.31 as glucurone, 0.12 as free glucuronic acid). After hydrolysis with 10% HCl, the chromatogram indicated 2 yellow spots at the positions corresponding to PAS and MAP when sprayed with the Ehrlich reagent and one brown spot at the position corresponding to glucurone when sprayed with aniline phthalate solution. When the solution was treated with 10% NaOH solution, the chromatogram indicated 2 spots corresponding to PAS and glucuronic acid. It may, therefore, be assumed that the spot at Rf 0.28 is that of PAS-glucuronic acid conjugate, although the position of conjugation has not been clarified.

The spot at Rf 0.54 was not that of MAP, on comparison with authentic MAP. The spots at Rf 0.24 and 0.2 are considered to be due to some additional metabolites of PAS, which were not characterized. These unknown spots are being studied farther.

The chromatogram of the urine of rabbits administered Ac-PAS indicated only one spot with the iron reagent, corresponding to authentic Ac-PAS.

Discussion

Acid solution of PAS decomposed rapidly upon heating to above 50° with the formation of MAP. Venkataraman[1] showed that there was no indication of decarboxylation in the rabbit and also in the incubation study of PAS with gastric and intestinal contents of a rabbit.

If this decarboxylation takes place to any considerable extent in the animal body, then the values obtained by the iron reaction should be much smaller than that obtained by the Ehrlich reaction. However, the results were not indicative of any significant difference between these two reactions. The paper chromatography of the urine showed that the position corresponding to MAP was negative to color reagents. It may, therefore, be considered that there is no considerble decarboxylation of PAS.

When PAS was administered in amimals, the excretion of Ac-PAS was very rapid but when Ac-PAS was administered, the excretion of unchanged Ac-PAS was very slow. This may be due to the difficult absorption of Ac-PAS, which is quite insoluble in acids and its solubility increases with an increase in pH. Only about 1% of Ac-PAS, of a 1.27 g./kg. dose, was deacetylated.

This is an interesting problem from the standpoint of detoxication mechanism. Free amino compounds excreted in the urine consisted of a mixture of free PAS and compounds conjugated with either hydroxyl or carboxyl group in the PAS molecule. The two compounds must give different intensities of color with the Ehrlich reagent. For example PAS and MAP showed a ratio of 1.47 to 1 in molar equivalents, but no corrections in calculations were made. These two compounds are theoretically possible but their effect on the color intensity is not known. In Tables I and II, free amine was calculated as PAS and the conjugated PAS as Ac-PAS.

Values thus obtained cannot be considered entirely reliable, but the values of total PAS and Ac-PAS, which were calculated as MAP and were converted to PAS or Ac-PAS by multiplying by the factors, are comparatively reliable.

Williams and Smith[10] showed that in rabbits, the glucuronic acid conjugate of *p*-acetaminobenzoic acid was much less than that of *p*-aminobenzoic acid. This suggests a possible relationship between glucuronic acid conjugation and acetylation. In the present investigation, the urine of rabbits administered PAS contained apparently increasing amounts of glucuronic acid output, but in the case of Ac-PAS, not at all or only small amounts. This indicates that PAS acetylated *in vivo* does not form the glucuronic acid conjugate or forms it with difficulty.

As the dose increased, the glucuronic acid output increased by degrees and the precentage of PAS conjugated with glucuronic acid decreased rapidly, reaching 2.3% at the 1 g./kg. dosage level and remained fairly constant at this figure. It appears likely therefore, that the source of acetylating agent is dried up temporarily by the rapid absorption of larger doses of PAS and as a result the acetylation is taken over by the glucuronic acid conjugation, which is also limited.

Urinary excretion of sulfates after abministrations of PAS and Ac-PAS indicated no increases in ethereal sulfate.

Further studies are necessary to establish the identity of unknown metabolites.

Summary

The metabolic fate of PAS and Ac-PAS in rabbits was studied comparatively. About 74% of the 1 g./kg. dose of PAS administered orally is excreted in the urine. Of this, 23~60% are unchanged, 17~42% as acetylated compounds, and 2~3% as a glucuronide. About 1% of a 1.27 g./kg. dose of Ac-PAS administered orally is deacetylated and 42~68% is excreted in the unchanged form. PAS glucuronide was identified by means of paper chromatography. There were three unknown metabolites containing a free amino group in the urine and no increase in ethereal sulfate nor considerable decarboxylation of either PAS or Ac-PAS were observed.

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