FURTHER STUDIES ON SOME ESTERS OF 4-AMINOSALICYCLIC ACID

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A series of alkyl and aryl esters of 4-aminosalicyclic acid (PAS) has been examined for protective effect in mice infected with *Mycobacterium tuberculosis* H37Rv, and the PAS blood levels after oral administration to mice, rats and rabbits studied. On the basis of these results, *p*-tolyl-4-aminosalicylate (*p*-tolyl PAS) was selected for further examination and has been shown to be of low toxicity in experimental animals and to produce moderately high and sustained PAS blood levels in man.

THE widespread use of 4-aminosalicylic acid (PAS) or its sodium salt as an auxiliary chemotherapeutic agent in the treatment of tuberculosis has directed much effort towards rendering this drug more palatable and less irritating to the gastrointestinal tract. A wide variety of pharmaceutical formulations such as tablets, granules and cachets have appeared which overcome the taste of PAS, but do little to mitigate its other side effects.

Calcium 4-benzamidosalicylate, a tasteless derivative of PAS which is hydrolysed in the body to PAS, has been shown[1,2] to be better tolerated than PAS and has recently become widely used as a substitute. However, the lower PAS blood levels produced with this substance led us to re-examine a series of esters of PAS, the tuberculostatic activity of which have been previously described[3] and further studies on these compounds are now reported.

EXPERIMENTAL METHODS

Antituberculosis activity. In vivo tests for tuberculostatic activity were carried out by the following method. Mice (18–20 g.) were injected intravenously with 0·3 ml. of a 10-day culture of *Mycobacterium tuberculosis* H37Rv and divided into groups of 10. One group served as an untreated control, and a second group received a diet containing 0·5 per cent sodium PAS. Other groups were given the test compounds suitably diluted in the diet. Median survival times (ST50) and 95 per cent confidence limits were determined using the graphical method of Litchfield[4] and chemotherapeutic activity expressed as the difference between the ST50s of treated and control animals (ΔST50).

PAS blood levels in rabbits. Drugs were given by stomach tube to rabbits, using two animals for each compound. Blood was taken 1, 2, 4 and 6 hours after drug administration.

PAS blood levels in mice. Drugs were given by stomach tube to groups of mice. Six animals were killed from each group at 1, 2, 4 and 6 hours after drug administration, and the pooled blood used for estimation of PAS and esterified PAS.

PAS blood levels in man. Drugs were given orally to healthy male volunteers, using three volunteers for each compound. Blood was taken 1, 2, 4 and 6 hours after drug administration.

PAS blood levels during conditions of a mouse survival test. Groups of mice were fed diets containing sodium PAS, phenyl PAS or *p*-tolyl PAS, at a dose equivalent to 0·5 per cent sodium PAS in the diet, for 14 days after which three animals from each group were killed at 2-hourly intervals over a 24-hour period, and the pooled blood taken for determination of PAS.

Tissue PAS levels in rats. Rats were pre-treated for 14 days with sodium PAS or *p*-tolyl PAS with a dose equivalent to 1 per cent sodium PAS in the diet. They were starved on the fourteenth night and on the following morning received a single dose of the appropriate drug equivalent to 500 mg./kg. sodium PAS, by stomach tube. Blood and tissue samples were taken 1 hour and 3 hours after drug administration. A control series of animals, which had not received the 14 days' pre-feeding, was included.

Blood PAS levels in humans after repeated drug administration. Twelve male volunteers were divided into three groups of 4, and treated for 15 days with sodium PAS, phenyl PAS, or *p*-tolyl PAS in three daily doses, using doses of drugs stoichiometrically equivalent to 3 × 60 mg./kg./day of sodium PAS. Blood samples were taken five times at 3-hourly intervals during the first and last days of treatment, and once on days 5, 9 and 12, and assayed for PAS content.

Total PAS. Estimations of total PAS, acid plus ester, were made by the method of Newhouse and Klyne[5], with the modification that diazotisations were at 0° to minimise errors due to small departures from the time schedule.

Esters of PAS in blood. The method described by Frederiksen, Jensen, Mørch, and Tybring[6] for the estimation of phenyl-4-aminosalicylate and phenyl PAS, in blood was not applicable to all the esters, and the following method was used. 2 ml. of blood was added to 6 ml. of 0·05N sodium hydroxide in a stoppered boiling tube. Ether, 30 ml., was added and the tube shaken for 10 minutes and the contents allowed to settle. 25 ml. of the ether layer was transferred to a 200 mm. B.24 stoppered boiling tube and evaporated to dryness in a stream of air. Water, 5 ml., and concentrated hydrochloric acid, 1·5 ml., were added, and after shaking to dissolve material adhering to the sides of the tube, diazotisation and coupling were carried out by the Newhouse and Klyne technique. After standing for 30 minutes, ethanol, 5 ml., was added and the colour density measured at 555 mμ. Standards were prepared by dissolving the ester, 8 mg., in ethanol, 100 ml. To 0·5 ml. of this solution was added 6 ml. 0·05N sodium hydroxide and 2 ml. normal blood, and the above procedure followed. Blank determinations were made using 2 ml. normal blood.

PAS in tissues. Animals were killed by decapitation and the liver and kidneys were freed from blood by perfusion with saline at 37°. This required about 50 ml. of saline. Lungs were similarly treated by perfusion into the vena cava and the heart. The organs after weighing were

homogenised with water to give a final concentration of 1 g. of tissue in 20 ml. of homogenate. 2·4 ml. of homogenate was shaken with 2 ml. of 10 per cent trichloroacetic acid and water, 4·5 ml., added. After 5 minutes, the mixture was filtered and the filtrate treated by the Newhouse and Klyne technique.

Total PAS derivatives in urine. Total urine excreted over 24 hours was collected into Winchesters containing a little toluene as preservative. The volume was measured and total PAS derivatives estimated after

TABLE I

INCREASE IN MEDIAN SURVIVAL TIME (△ST50) IN MICE INFECTED WITH *M. TUBERCULOSIS* H 37RV

(95 per cent confidence limits in parentheses)

Compound	Dose	△ ST50 (days)
Sodium PAS	0·5 per cent in diet	+ 17 (10–24); + 13 (9–17); + 22 (14–32)
Phenyl PAS	≡ 0·5 per cent Na PAS in diet	+ 1 (0–4); + 18 (8–31); + 4 (0–8)
p-Tolyl PAS	,,	+ 15 (8–22); + 13 (9–17); + 19 (11–28); + 23 (17–29
o-Tolyl PAS	,,	+ 7 (1–13)
m-Tolyl PAS	,,	+ 6 (1–12); + 12 (5–19)
Ethyl PAS	,,	−3 (−8–+ 2); −3 (−8–+ 2)
β-Hydroxyethyl PAS ..	,,	+ 5 (0–10); + 7 (1–13)

TABLE II

PAS BLOOD LEVELS (μG./ML.) IN RABBITS FOLLOWING AN ORAL DOSE EQUIVALENT TO 500 MG./KG. Na PAS $2H_2O$

Compound	1 hr.		2 hr.		4 hr.		6 hr.	
	Total PAS	Ester	Total PAS	Ester	Total PAS	Ester	Total PAS	Ester
Sodium PAS	78	0	53	0	17	0	6	0
	86	0	39	0	25	0	12	0
Phenyl PAS	6	1	9	0	14	1	11	1
	5	2	2	1	7	1	6	1
p-Tolyl PAS	3	0	3	0	21	0	13	0
	14	1	12	0	13	0	6	0
m-Tolyl PAS	6	3	5	0	7	1	6	2
	10	1	11	0	22	0	19	0
o-Tolyl PAS	11	3	17	0	26	1	13	2
	15	1	—	0	25	2	26	1
Ethyl PAS	41	1	45	1	29	0	20	0
	26	1	16	0	13	0	5	0
β-Hydroxyethyl PAS ..	67	2	53	0	14	0	3	0
	40	5	20	1	17	0	5	0

hydrolysis at 40° using the method of Way, Smith, Howie, Weiss and Swanson[7].

Chromatographic examination of urine. Urine was applied directly to Whatman No. 1 paper and developed with *n*-butanol:ethanol:2N ammonium hydroxide, 4:1:5 by the ascending technique. Spots were detected either by dipping in Ehrlich's reagent for free amines, or by spraying with ferric nitrate solution.

Acute toxicities. Acute toxicity determinations were made in rats and mice, drugs being given orally by stomach tube.

Chronic toxicity of p-*tolyl PAS.* Forty male rats (70 to 100 g.) were divided into four equal groups. One group served as a control and the others received *p*-tolyl PAS, administered in the diet, at doses of 250, 625, and 1560 mg./kg. The animals were weighed twice weekly and growth curves plotted for each group. After 14 weeks, determinations of blood urea, glucose, and haemoglobin, red cell, white cell, differential white cell, and reticulocyte counts, urine albumen and glucose were

TABLE III

PAS BLOOD LEVELS (μG./ML.) IN MICE FOLLOWING AN ORAL DOSE EQUIVALENT TO 500 MG./KG. Na PAS $2H_2O$

Compound	1 hr.		3 hr.		5 hr.	
	Total PAS	Ester	Total PAS	Ester	Total PAS	Ester
Sodium PAS	63	4	0	0	0	0
Phenyl PAS	17	6	4	0	4	0
p-Tolyl PAS	14	2	2	1	6	1
Ethyl PAS	39	9	17	3	8	1
3-Hydroxyethyl PAS	132	36	17	1	2	2

TABLE IV

PAS BLOOD LEVELS* (μG./ML.) IN MAN AFTER AN ORAL DOSE EQUIVALENT TO 80 MG./KG. Na PAS $2H_2O$

Compound	1 hr.	2 hr.	4 hr.	7 hr.
Sodium PAS	45	55	17	3
	96	67	28	4
	80	48	12	0
Phenyl PAS	8	6	4	3
	19	11	5	3
	12	4	3	3
p-Tolyl PAS	19	11	2	0
	12	7	6	3
	25	14	5	0

* Blood levels of esterified PAS were also determined and found to be zero at all times.

carried out. After 17 weeks the animals were killed and specimens of liver, lung, heart, stomach, kidney, spleen, thyroid and adrenals taken for histological examination.

RESULTS

Chemotherapeutic tests. Table I shows the results of mouse survival tests on a series of PAS esters, compared with sodium PAS. It is apparent that *p*-tolyl-4-aminosalicylate (*p*-tolyl PAS)[8,9] displays consistently similar protection to that of sodium PAS. Other aryl esters, and the β-hydroxyethyl ester appeared to be considerably less active than sodium PAS, and the ethyl ester was inactive. This latter result is in agreement with previous reports of the lack of *in vivo* antituberculosis effect of alkyl esters of PAS. As it seemed likely that the protective effect of PAS esters was due to PAS produced by hydrolysis in the body, it was of

interest to determine whether the superior protective effect of *p*-tolyl PAS was due to PAS blood levels higher than those achieved with the other esters.

TABLE V

PAS BLOOD (μG./ML.) AND TISSUES (μG./G.) LEVELS IN RATS AFTER A SINGLE ORAL DOSE OF 500 MG./KG. Na PAS $2H_2O$ OR 576 MG./KG. *p*-TOLYL PAS, WITH AND WITHOUT 14 DAYS PRE-FEEDING AT A LEVEL OF 1·0 PER CENT IN DIET

Tissue	PAS levels in μg./ml. or μg./g.			
	1 hr. control	1 hr. pre-fed	3 hr. control	3 hr. pre-fed
	Sodium PAS			
Blood	167 174	152 156	23 12	100 57
Kidney	73 199	485 417	193 41	315 167
Liver	123 —	100 88	16 10	60 40
Lung	14 22	103 48	7 6	30 14
	p-Tolyl PAS			
Blood	19 14	51 51	7 4	51 30
Kidney	51 51	116 181	20 16	75 93
Liver	12 10	36 54	4 3	30 48
Lung	2 2	6 26	0 0	20 —

TABLE VI

PAS BLOOD LEVELS (μG./ML.) IN HUMAN VOLUNTEERS FOLLOWING ORAL ADMINISTRATION OF DOSES EQUIVALENT TO 3 × 60 MG./KG. Na PAS $2H_2O$/DAY FOR 15 DAYS

(Drugs administered at 08.00, 13.00 and 18.00 hours)

Subject	Drug	Day 1					Day 5	Day 9	Day 12	Day 15					Day 16
		09.00	12.00	15.00	18.00	21.00	15.00	15.00	15.00	09.00	12.00	15.00	18.00	21.00	09.00
M.W.	Sodium*	46	3	37	0	13	36	—	36	51	14	43	21	61	0
M.M.	PAS	49	8	22	0	14	—	43	—	48	19	42	10	27	0
K.T.		54	7	36	1	26	25	16	64	70	12	48	9	36	0
mean of the group		50	6	32	0	18				56	15	44	13	41	
R.R.†	Phenyl	27	4	11	1	19	18	—	22	—	—	—	—	—	—
G.V.B.	PAS	13	1	0	0	0	11	10	25	12	1	20	4	16	0
R.H.		13	4	11	3	11	8	30	32	14	14	27	11	24	0
L.M.		11	0	5	1	8	14	13	19	16	1	17	9	27	0
mean of the group		16	2	7	1	9				14	5	21	8	22	
F.J.	*p*-Tolyl	4	6	13	1	10	23	41	75	11	25	48	38	46	0
A.A.	PAS	12	7	22	3	9	57	32	52	24	17	29	10	14	0
W.S.		22	3	31	2	7	35	35	42	15	6	38	9	36	0
G.A.P.		17	2	5	3	8	26	6	5	16	19	16	29	43	5
mean of the group		14	4	18	2	8				16	17	33	21	35	

* One of the sodium PAS group left the trial on Day 2 due to illness.
† R.R. was withdrawn from the trial on Day 13 due to gastrointestinal side-effects of the drug.

Biochemical. Tables II, III and IV show the blood levels of both total PAS, acid and ester, and esterified PAS after oral administration of single doses of sodium PAS, and PAS esters to mice, rabbits, and humans. In all three species, sodium PAS is rapidly absorbed and excreted giving a

peak level approximately 1 hour after administration, the two alkyl esters show a similar pattern. Aryl esters give much lower levels in all the species, and in rabbits show a different pattern, the PAS levels rising gradually over the 6-hour observation period. With the exception of the

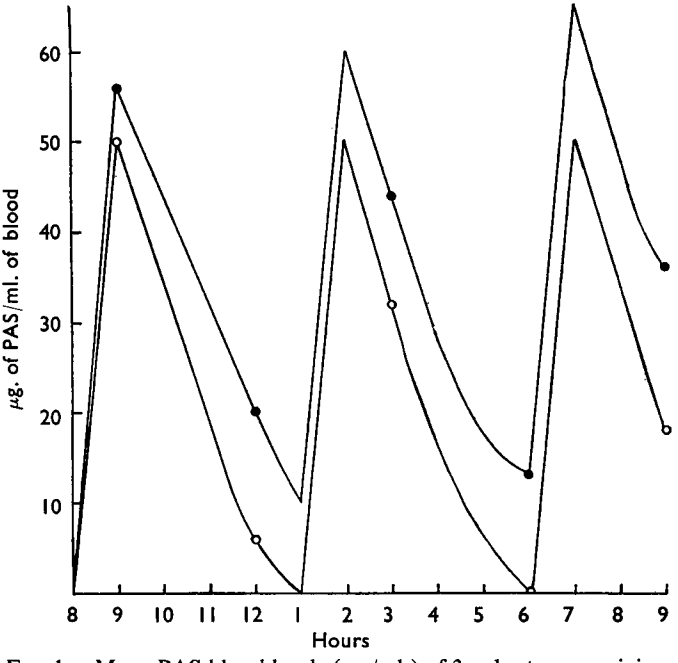


FIG. 1. Mean PAS blood levels (μg./ml.) of 3 volunteers receiving Sodium PAS. "Day 1" and "Day 15".
○ = Day 1. ● Day 15.

two alkyl esters in mice, the blood contains little or no esterified PAS, indicating rapid hydrolysis; in subsequent work, total PAS only was measured.

The blood PAS levels under conditions of mouse survival tests were very low, ranging from 0–6 μg./ml. Mean values for the three drugs examined were, sodium PAS, 3·0 μg./ml., phenyl PAS, 2·5 μg./ml., and *p*-tolyl PAS, 2·6 μg./ml.

Table V gives the PAS levels in blood, kidney, liver and lung of groups of rats following a single oral dose of sodium PAS or *p*-tolyl PAS, with and without 14 days pre-feeding of the appropriate drug. PAS levels were markedly higher in the pre-fed groups than in the control animals, and these results, together with the report of Torning[10] that blood PAS levels in patients treated daily with phenyl PAS steadily increased over a 3-week period, led us to investigate the PAS blood levels produced by some of these esters after repeated administration to man according to current clinical practice.

The results (Table VI) show that PAS blood levels were appreciably higher on day 15 than on day 1, and that the differences between mean

values for the 2 days were significant ($P<0.05$), except for the 0900 hour readings where, in all three groups, the increases were not significant. By plotting mean values for day 15 and using suitable interpolated points,

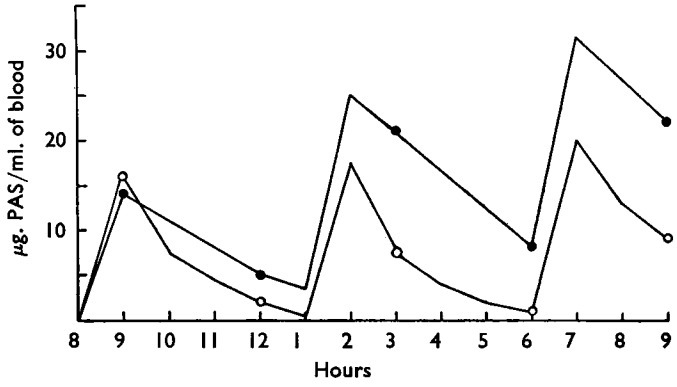


FIG. 2. Mean PAS blood levels (μg./ml.) of 4 volunteers receiving phenyl PAS. "Day 1" and "Day 15".
○ = Day 1. ● = Day 15.

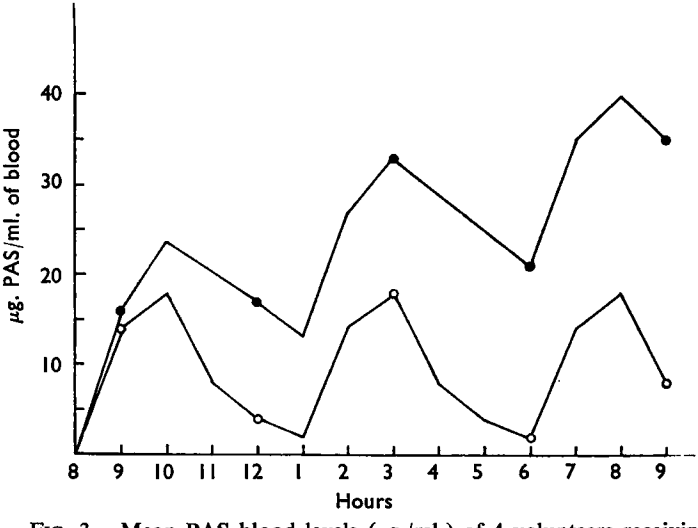


FIG. 3. Mean PAS blood levels (μg./ml.) of 4 volunteers receiving *p*-tolyl PAS. "Day 1" and "Day 15".
○ = Day 1. ● = Day 15.

an average blood level curve was constructed for each drug, these are shown in Figures 1, 2 and 3. By measurement of the area underneath the curves, an overall mean blood level for each drug during the observation period has been calculated, sodium PAS, 33 μg./ml., phenyl PAS, 16 μg./ml., and *p*-tolyl PAS, 24 μg./ml.

Twenty-four hour urine samples were collected from all subjects during the tenth day to determine the extent of absorption. Mean values for the

three groups, expressed as percentage of daily dose, were sodium PAS, 83 per cent (range 79 to 85 per cent), phenyl PAS, 63 per cent (range 43 to 74 per cent), and *p*-tolyl PAS, 68 per cent (range 61 to 88 per cent). Chromatographic examination revealed in all instances the presence of PAS, acetyl PAS and 4-aminosalicyluric acid, indicating a similar metabolic pattern for the PAS fragment of all three drugs.

Acute toxicity. Median lethal doses (oral) were in mice ethyl PAS, 1·5 g./kg., β-hydroxyethyl PAS, 2·0 g./kg., and *p*-tolyl PAS, about 10 g./kg. Mice receiving 1·0 g./kg. and above of β-hydroxyethyl PAS or ethyl PAS showed macroscopic pathological changes in liver, kidney and spleen.

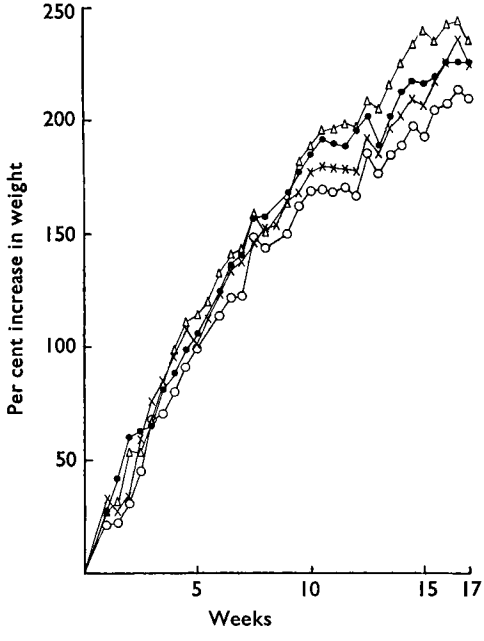


FIG. 4. The effect of *p*-tolyl PAS on growth of rats (10 animals per group).
○ = 1563 mg./kg. ● = Control
× = 625 mg./kg. △ = 250 mg./kg.

Ethyl PAS, and β-hydroxyethyl PAS in rats gave no deaths on doses up to 8·0 g./kg. orally, but all the animals except those receiving 1·0 g./kg. ethyl PAS showed pathological changes in liver, kidneys and spleen.

Chronic toxicity of p-*tolyl PAS.* Growth curves (Fig. 4) show that those animals receiving the highest dose, 1560 mg./kg., had a slightly reduced growth rate compared with the controls. Statistical analysis showed this decrease to be not significant. Blood urea and glucose, and urine albumen and glucose were within the normal range. Blood haemoglobin and red cell counts showed no difference from the controls when analysed statistically, but with the animals on the highest dose the white cell count (mean, 10,150/cu. mm.) was slightly less than the control value (mean, 13,300/cu. mm.), and the reticulocyte count (mean, 1·2 per cent) was slightly greater than the control value (mean, 0·5 per cent). Animals receiving the two lower doses showed no significant alteration in white cell or reticulocyte counts. A preliminary examination of sections from the animals receiving 1560 mg./kg. has shown no evidence of tissue damage, but the complete histological findings are not yet available.

DISCUSSION

Examination of the results of mouse survival tests (Table I) together with those of the chemically determined PAS blood levels (Tables II, III

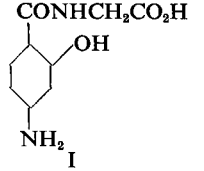
IV) shows some surprising features. Aryl esters of PAS give low PAS blood levels yet many show a marked protective effect on infected mice. Alkyl esters (ethyl and β-hydroxyethyl) give high PAS blood levels, but give less protection. It was thought that the first of these observations could be explained by postulating that, during the continuous dosage conditions of the mouse survival test, build up of PAS occurred, leading to higher blood levels after several days. The results indicate that this is not so, but do show the low PAS blood levels required for a protective effect in this test.

The low protective effect of ethyl and β-hydroxyethyl PAS confirms previous reports[3,11] on the inactivity of the alkyl esters of PAS *in vivo* and may be ascribed to toxic effects masking any protection afforded by the liberated PAS. Examination of mice receiving doses equivalent to those used in the mouse survival test (0·5 per cent in diet = about 1·25 g./kg.) showed pathological changes in liver, kidneys and spleen.

The effect of pre-feeding rats for 14 days with sodium PAS or *p*-tolyl PAS on the PAS blood and tissue levels after a single oral dose, was to increase considerably the PAS levels. The fact that the decline from 1-hour level to 3-hour level was much less in the pre-fed animals than in the controls may indicate some saturation of the enzymes responsible for the metabolic transformation of PAS, or maybe a block of excretion. The reason for the similarity in the 1-hour levels in the two sodium PAS groups may be that, with this drug, blood levels at 1 hour are controlled largely by its very rapid absorption rate, and any alteration in excretion rate would have little effect. With the more slowly absorbed *p*-tolyl PAS, the 1-hour level is markedly affected by excretion rate, and when this is reduced by pre-feeding, the 1-hour level is enhanced.

The blood level increases obtained in human volunteers are also best accounted for on the basis of a decreased rate of excretion, the difference between day 15 and day 1 blood levels being greatest, 3, 4 and 5 hours after a dose, and tending to increase throughout the day (Figs. 1, 2 and 3). A further experiment designed to compare PAS blood levels after prolonged administration of calcium 4-benzamidosalicylate and sodium PAS has been recently reported[12] in which similar blood level increases were demonstrated. These results, although based on small numbers are nevertheless significant and indicate that blood level determinations with drugs intended for long-term administration should be made after a period of continuous treatment, and not after a single dose or a series of doses spaced over one day.

Although the mechanisms by which lowered excretion rate of PAS occurs has not been explained, the similarity in chemical structure of the PAS metabolite 4-aminosalicyluric acid (I) and the known renal-blocking



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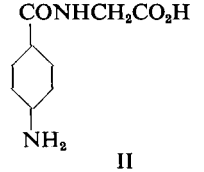

II

drug, 4-aminohippuric acid (II) suggests that 4-aminosalicyluric acid may also be acting as a renal-blocking agent and thereby leading to an increased blood concentration of its precursor, PAS.

From the data reported in this paper, *p*-tolyl PAS is shown to be of low toxicity, only minor toxic symptoms appearing after a dose of 1560 mg./kg. daily for 3 months in rats. This, together with the fact that it is tasteless and produces sustained PAS blood levels approaching those achieved with equivalent doses of sodium PAS, indicates that this substance fulfils many of the criteria for a clinically acceptable PAS derivative.

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After Mr. Drain presented the paper there was a DISCUSSION. The following points were made.

The discussion revealed the lack of specificity of the methods of estimation, and the doubtful validity of blood PAS levels. The compounds were soluble to the extent of 10 mg./100 ml. and would probably hydrolyse very slowly at pH 7. It was suggested that steric factors and the configuration of the molecules might account for the differences in activity of the aryl and alkyl esters.