In vitro and *in vivo* analysis of the PABA test compared with the Lundh test—influence of intraluminal pH

F J HOEK, G T B SANDERS, A TEUNEN, AND G N J TIJTGAT

From the Departments of Internal Medicine and Gastroenterology, University Hospital Wilhelmina Gasthuis, Amsterdam, the Netherlands

SUMMARY   Lundh test and PABA test results were compared in 50 patients. In the resulting correlation curve three areas of interest were differentiated: (1) an area with mean tryptic activity, found in the Lundh test, below 4 U/ml, where an abnormal PABA test result was also seen; (2) an intermediate area from 4 to 9 U/ml, where PABA test results may be found in discordance with the Lundh test; (3) the normal level, where PABA excretion was shown to be independent of intestinal chymotrypsin activity. In experiments *in vitro* on the kinetics of the hydrolysis of BTPABA by chymotrypsin the profound influence of the pH on Km and Vmax was shown. This influence of the pH explains why in the intermediate area of pancreatic dysfunction normal PABA test results were found in a number of cases. A higher mean pH level of the Lundh test aspirates was found for patients with a normal PABA test result than for patients with an abnormal PABA test.

Recently a number of reports[1-5] have appeared on the merits of a new pancreatic function test. The test uses a substrate specific for chymotrypsin—namely, N-benzoyl-L-tyrosyl-p-aminobenzoic acid (BTPABA). The p-aminobenzoic acid (PABA), moiety is, when it is split off, rapidly absorbed by the intestinal epithelium by a passive process and, almost completely in the acetylated form, excreted in the urine. The amount of PABA in the urine is used as an index of pancreatic function. Usually a six hour urine collection period is used after administration of the test substance together with a Lundh test meal or a glucose solution, because this is the shortest collection period giving good discrimination between patients and normal subjects.[2 3] In the literature this test is compared with the Lundh test,[2-4] the secretin-pancreozymin test,[6 7] faecal chymotrypsin activity[5 8-11] and faecal fat[9 10] and is said to give a good correlation with these tests. This comparison, however, was mostly made in normal subjects and in unequivocal cases of moderate to severe pancreatic insufficiency.

In order to document its clinical usefulness in a wide spectrum of suspected pancreatic abnormalities in which a routine Lundh test was performed, an additional PABA test was done for comparison of discriminatory accuracy. In a number of cases a discrepancy was observed between the two tests, revealing a normal PABA test in the presence of a mildly abnormal Lundh test. This difference made us investigate more thoroughly the kinetics of some of the processes which occur after the oral administration of BTPABA.

Methods

Per test 1 g[11] BTPABA was administered in tablet form together with 150 ml of a Lundh test meal. From this time urine was collected for at least six hours, if possible in collection periods of one hour. Oral water intake during the test period was encouraged. The Lundh test meal contains 6% (w/v) fat, 5% (w/v) protein, and 15% (w/v) carbohydrate in water.

In a small number of volunteers an equimolar amount of free PABA (340 mg) was administered, either in combination with 150 ml of the Lundh test meal or with water only.

The Lundh test was performed in the usual way,[12] with one 30 minute collection period before and four 30 minute collection periods after administration of 300 ml of the test meal. When Lundh test

and PABA test were performed simultaneously, only 150 ml of the test meal was given. In the intestinal aspirates pH and trypsin activity were measured. Assay for trypsin was performed on a Radiometer autotitrator module, with a pH stat method using N-benzoyl-L-arginine ethyl ester hydrochloride (BAEE) as a substrate.[13] With this method our lower limit of normal for the mean trypsin activity (MTA) is 8·6 U/ml, which is in agreement with the value of 9·6 U/ml reported in the literature.[12]

In vitro determination of the kinetics of proteolysis of BTPABA by chymotrypsin was performed with different concentrations of the substrate, ranging from 0·25 to 9·9 × 10^{-3} mol/l, in 0·1 mol/1 phosphate (pH 7·8 and 7·1) or citrate-phosphate buffer (pH 6·1 and 5·4). To 1·8 ml of the substrate solution, preincubated at 37°C, 0·2 ml of pooled intestinal contents with a trypsin activity of 10 U/ml was added, giving a final activity of 1 U/ml. After five to 30 minutes, with intervals of five minutes, 0·2 ml of the incubated solution was pippetted into 5 ml 4N HCl in order to stop the reaction and to determine the amount of liberated PABA. PABA was determined with the Bratton and Marshall method.[14 15] The BTPABA concentration in intestinal fluid was determined from the amount of liberated PABA after hydrolysis of the peptide with 5 volumes 6N HCl for one hour at 100°C in a closed vessel.[1]

Results

In order to determine accurately the kinetics of the urinary excretion of PABA present in the body after ingestion of BTPABA with 150 ml Lundh meal, 20 normal controls were investigated. Urine was collected in one hour portions for eight hours. Mean values and SD were determined for the individual portions and for the cumulative urinary PABA excretion (Fig. 1), resulting for the six hour collection period in a normal range ($\bar{x} \pm 2SD$) of 55·2 to 92·8%. When the depletion of the PABA present in the body was plotted on semi-log graph paper, a straight line was obtained after an initial lag phase. This did suggest that in normal subjects the urinary excretion of PABA depended on a process with first order kinetics. From this disappearance curve, it was possible to calculate a $T\frac{1}{2}$ value for the urinary excretion rate, ignoring the initial lag period, probably to a large extent corresponding to stomach emptying. For the 20 normal controls a $T\frac{1}{2}$ value of 118±55 min ($\bar{x} \pm 2SD$) was calculated. The PABA present in the urine was mainly in the acetylated form, as preceding acid hydrolysis was necessary for proper determination.
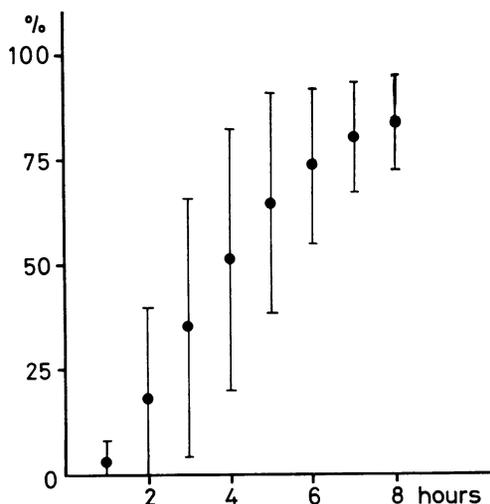

Fig. 1 *Cumulative urinary PABA excretion in % of the applied dose of BTPABA as a function of time. Results of 20 normal controls (mean ±2SD).*

Then, in five normal subjects urinary PABA excretion was followed in the same way after ingestion of an equimolar amount of free PABA, also with 150 ml of Lundh test meal. A very similar excretion pattern was obtained, apart from a shorter initial lag phase. Semi-log plotting gave as value for $T\frac{1}{2}$ 108±22 min ($\bar{x} \pm 2SD$). Ingestion of free PABA with water instead of Lundh test meal resulted in a much faster PABA excretion. In contrast with the normal situation, where only small amounts of unconjugated PABA could be detected in the urine—that is, without previous acid hydrolysis—25–30% of the excreted PABA—mainly in the first two hours—reacted directly after oral administration of free PABA in water.

The results of PABA and Lundh test for 50 consecutive patients presenting with pancreatic, small intestinal, liver, or biliary complaints, were compared as summarised in Fig. 2. The time lapse between PABA and Lundh test was usually only a few days and never exceeded two weeks. In accordance with the literature[3 11] a lower limit of normal of 50% is used, which is somewhat lower than our own control group, consisting only of rather young healthy persons. From this correlation curve three areas of interest could be differentiated. First, an area with a mean intestinal trypsin activity, as measured in the Lundh test, lower than 4 U/ml, where also an abnormal PABA test result was found, thereby correlating with the low pancreatic enzyme concentration.
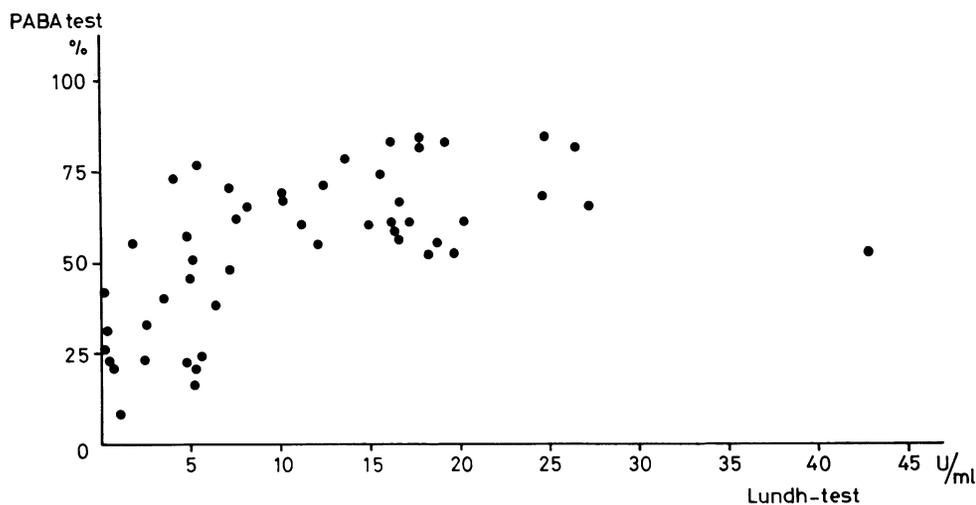
Secondly, the intermediate region of mild to

Fig. 2 *Comparison of PABA test and Lundh test results for 50 patients.*

moderate pancreatic insufficiency, with a MTA ranging from about 4 to 9 U/ml. In this area normal PABA test results were found in a substantial number of cases, in discordance with the abnormal Lundh test results.

The third area contained normal Lundh test findings in correspondence with normal PABA test results. In this normal range the percentage of PABA excreted in a six hour urine portion was apparently independent of the MTA. In order to explain the discrepancies in the second area, further

in vitro determinations of the kinetics of the hydrolysis of BTPABA by chymotrypsin in human duodenal fluid were performed. Determination of the Km resulted in a value of $2 \cdot 2 \times 10^{-3}$ mol/l at pH 7·8, which corresponds with the values of $2 \cdot 9 \times 10^{-3}$ mol/l and $1 \cdot 28 \times 10^{-3}$ mol/l reported in the literature.[16][17] Lowering of the pH to levels which are more regularly found in small intestinal aspirates resulted in substantial lowering of the values for Km and Vmax as illustrated in Fig. 3. The limited solubility of the BTPABA in acid media prevented
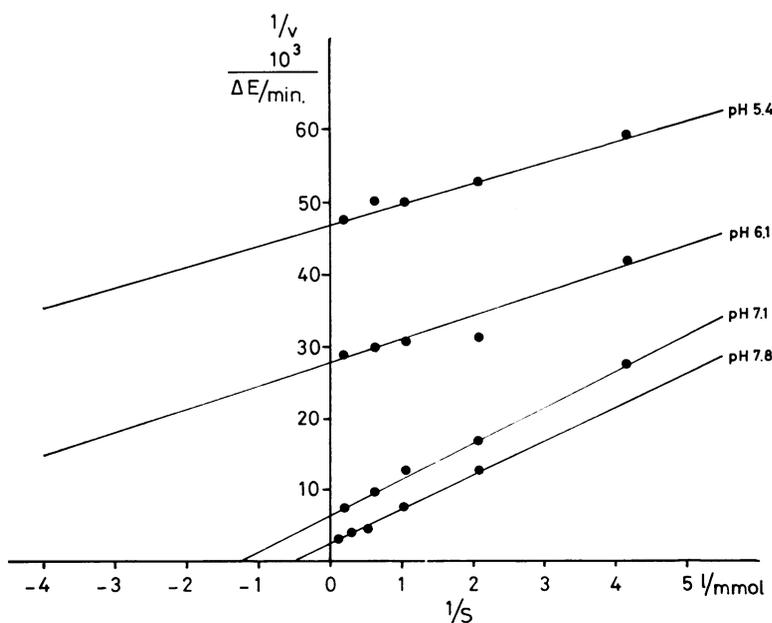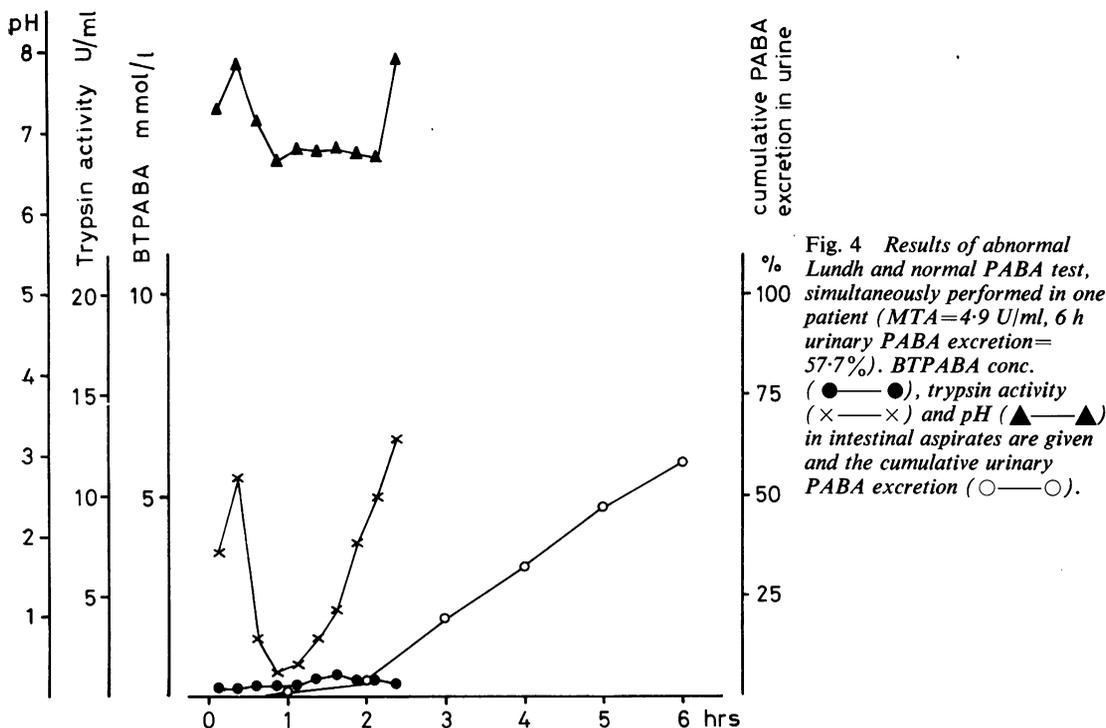

Fig. 3 *Lineweaver-Burk plot of the hydrolysis of BTPABA by chymotrypsin in human duodenal fluid at different pH values.*

Fig. 4   *Results of abnormal Lundh and normal PABA test, simultaneously performed in one patient (MTA=4·9 U/ml, 6 h urinary PABA excretion= 57·7%). BTPABA conc. (●——●), trypsin activity (×——×) and pH (▲——▲) in intestinal aspirates are given and the cumulative urinary PABA excretion (○——○).*

the determination of Km and Vmax at a pH lower than 5·4. In four cases—two normal, one frankly abnormal, one mildly abnormal—Lundh test and PABA test were performed simultaneously, with determination of pH, trypsin activity, and BTPABA concentration in 15 minute portions of intestinal aspirate. PABA test results were corrected for the amount of BTPABA recovered from the intestinal aspirates. BTPABA concentration in the intestine was always in the millimolar range.

In the patient (Fig. 4) with a mildly abnormal Lundh test (MTA=4·9 U/ml), a normal PABA test result was calculated. The pH of the aspirated intestinal contents had a mean value of 7·0. The mean intestinal pH measured in the Lundh test aspirates for 15 normal subjects, having a normal PABA test, was $6·07 \pm 0·61$ ($\bar{x} \pm SD$). In five mildly abnormal Lundh tests where an abnormal PABA test was also found, the mean intestinal pH was $5·90 \pm 0·42$. In contrast with these results in eight mildly abnormal Lundh tests, where a normal PABA test was found, the mean intestinal pH was $6·54 \pm 0·47$. Even in patients with normal pancreatic function, an influence of intraluminal pH on the PABA test result could be shown. In these normal subjects the result of the PABA test appeared to be independent of the MTA as found in the Lundh test (Fig. 2). The percentage of PABA recovered

from the urine in these normal subjects showed, however, a correlation—although weak—with the mean intestinal pH found in the Lundh test ($r=0·58$; $P < 0·05$).

Discussion

Comparative study of the PABA test and Lundh test showed that urinary PABA excretion was directly dependent on the chymotrypsin activity in the intestinal fluid only in patients with more severely disturbed pancreatic function (Fig. 2). The change from chymotrypsin dependent PABA excretion in the urine to a PABA excretion determined by other processes resulted in a second, intermediate, area where normal values for the PABA test could be found in discordance with the abnormal results of other pancreatic function tests. False normal values have also been reported by Bornschein[17] and Harada et al.[7] The third area, which could be distinguished, was the normal PABA range, showing an obvious independence of the enzyme activity (Fig. 2). This independence of the pancreatic function in the normal range becomes visible also in the shape of the curves found by Arvanitakis et al.[4] and Gyr et al.[3] comparing the PABA test with the Lundh test, and by Imamura et al.[6] and Harada et al.[7] comparing the PABA test with the pancreozymin-secretin test.

We did not investigate which process is rate-limiting at normal intestinal chymotrypsin activity. Possibilities are stomach emptying, transport across the intestinal wall, acetylation, and conjugation presumably in the liver, or urinary excretion. From these, stomach emptying can probably be excluded, because we showed that ingestion of the same amount of free PABA, not together with a Lundh test meal but with water only, resulted in faster urinary PABA excretion, whereby a substantial amount of non-acetylated PABA was recovered, mainly in the one and two hour urine samples.

Apparently, then, the maximal acetylation capacity is exceeded as a consequence of faster intestinal uptake, presumably because of more rapid stomach emptying with an aqueous PABA solution compared with the protein and lipid containing Lundh test meal.[18]

The simultaneous intake of a Lundh test meal not only provides physiological stimulation of the pancreas, but also slower gastric emptying, resulting in a more gradual delivery of substance to the intestine and thereby more gradual appearance of PABA in the urine, which then occurs almost completely in the acetylated form.

At the acid pH of the gastric juice, BTPABA is insoluble in contrast with free PABA, which does not precipitate at that pH. This could be another factor leading to more gradual delivery of PABA to the intestinal wall for resorption. That this may result in a faster stomach evacuation for free PABA can be deduced from the semi-log plots, where a shorter lag phase is found before the rate-limiting first order process.

In the intermediate range of mild to moderate pancreatic insufficiency, there is a confluence of two slow processes with velocities of the same order of magnitude: PABA excretion dependent on chymotrypsin activity and on other factors. The reaction conditions can determine which process will be the slowest. Therefore, we see in this range in a number of cases not only an abnormal Lundh test in combination with an abnormal PABA test, but also in a significant number of patients a discrepancy between Lundh and PABA test.

Kinetic experiments *in vitro* clearly show a profound influence of the pH on Vmax as well as Km of chymotrypsin with PABA as substrate (Fig. 3). This pH influence becomes rather important, because duodenal pH can vary substantially—even in normal subjects—between 4 and 8. The average pH of 6·07 found in Lundh aspirates of our normal controls is substantially different from the pH optimum of 7·8 of chymotrypsin.

The combined Lundh and PABA tests, performed in a small number of persons, show that the BTPABA concentration in the duodenum is in the millimolar range (Fig. 4), the range of Km, and that therefore the turnover rate at higher pH of the intestinal fluid will be about half-maximal. At lower pH, where the Km is 10 times lower (Fig. 3), the turnover rate will reach approximately the maximal rate for the corresponding pH. The hydrolysis of 1 g BTPABA would then be completed in 120 minutes at pH 7·8 and in about 1130 minutes at pH 5·4 according to our *in vitro* experiments, in which the final trypsin activity was 1 U/ml.

Comparison of these figures with a T½ of 118 minutes for urinary PABA excretion shows that the *in vitro* kinetic studies also make it plausible that only in mild to moderate pancreatic insufficiency areas intestinal chymotrypsin activity becomes decisive for the amount of PABA excreted in the urine. Combined performance of Lundh and PABA test gave in one patient with moderate pancreatic dysfunction a urinary PABA excretion in the normal range. The corresponding pH of the intestinal aspirates (mean pH 7·0) was relatively high, explaining the 'normal' PABA excretion. Other cases, in which a discrepancy does exist between the results of the Lundh and the PABA test, also show a higher mean pH of the intestinal fluid, compared with normal subjects or to patients with a moderately abnormal Lundh and abnormal PABA test.

Surprisingly, even in normal subjects, a correlation —although weak—is found between the result of the PABA test and the intestinal pH, where T½ for urinary PABA excretion does not reflect an intestinal process. We must therefore assume that the influence of gastric emptying and of the pH of the intestinal contents on the delivery of free PABA to the intestinal wall is important enough to be reflected in the correlation found. Overall, there is an acceptable correlation between the Lundh test and the PABA test. In situations of moderately low luminal trypsin (and presumably chymotrypsin) activity, intestinal pH becomes of major importance in determining the velocity of BTPABA hydrolysis. The higher the luminal pH, the less abnormal the urinary PABA excretion will be.

References

[1]Imondi AR, Stradley RP, Wolgemuth R. Synthetic peptides in the diagnosis of exocrine pancreatic insufficiency in animals. *Gut* 1972; **13**: 726–31.
[2]Gyr NE. *Tests of exocrine pancreatic function.* Bern: Hans Huber, 1975: 92–112.
[3]Gyr K, Stalder GA, Schiffmann I, Fehr C, Vonder-schmitt D, Fahrlaender H. Oral administration of a

chymotrypsin-labile peptide—a new test of exocrine pancreatic function in man (PFT). *Gut* 1976; **17**: 27–32.

[4]Arvanitakis C, Greenberger NJ. Diagnosis of pancreatic disease by a synthetic peptide (N-benzoyl-L-tyrosyl-p-amino benzoic acid): a new test of exocrine pancreatic function. *Lancet* 1976; **1**: 663–6.

[5]Bornschein W, Goldmann FL, Dressler J. Diagnostik der exokrinen Pankreasinsuffizienz mit einem synthetischen chymotrypsin-spezifischen Peptid. *Klin Wschr* 1978; **56**: 197–205.

[6]Imamura KI, Nakamura T, Miyazawa T, *et al.* Oral administration of chymotrypsin labile peptide for a new test of exocrine pancreatic function (PFT) in comparison with pancreozymin-secretin test. *Am J Gastroenterol* 1978; **69**: 572–8.

[7]Harada H, Mishima K, Shundo T, *et al.* Exocrine pancreatic function test by a synthetic peptide. *Am J Gastroenterol* 1979; **71**: 45–52.

[8]Sacher M, Kobsa A, Shmerling DH. PABA screening test for exocrine pancreatic function in infants and children. *Arch Dis Childh* 1978; **53**: 639–41.

[9]Nousia-Arvanitakis S, Arvanitakis C, Desai N, Greenberger NJ. Diagnosis of exocrine pancreatic insufficiency in cystic fibrosis by the synthetic peptide N-benzoyl-L-tyrosyl-p-aminobenzoic acid. *J Pediatr* 1978; **92**: 734–7.

[10]Bühler H, Ammann R. PABA Test versus Chymotrypsin und Fettbestimmung im Stuhl in der Pankreasfunktions-diagnostik. *Schweitz Med Wschr* 1979; **109**: 597–8.

[11]Thienhaus R, Niederau C. Die Untersuchung der exokrinen Pankreasfunktion mit N-Benzoyl-L-Tyrosyl-p-Aminobenzoesäure. *Z Gastroenterol* 1979; **17**: 187–94.

[12]Cook H Bramwell, Lennard-Jones JE, Sherif SM, Wiggins HS. Measurement of tryptic activity in intestinal juice as a diagnostic test of pancreatic disease. *Gut* 1967, **8**: 408–14.

[13]Wiggins HS. Simple method for estimating trypsin. *Gut* 1967; **8**: 415–6.

[14]Bratton AC, Marshall EK: A new coupling component for sulfonamide determination. *J Biol Chem* 1939; **128**: 537–50.

[15]Annino JS, Senesky D. Sulfonamides. In: Seligson D, ed. *Standard methods of clinical chemistry*, vol. 3. New York and London: Academic Press, 1961; 200–6.

[16]Imondi AR, Stradley RP, Butler ER, Wohlgemuth RL. A method for the assay of chymotrypsin in crude biological materials. *Anal Biochem* 1973; **54**: 199–204.

[17]Bornschein W. Der Peptid-PABA-Test. Methodische und klinische Untersuchungen mit einem neuen indirekten Pankreasfunktionstest. *Fortschr Med* 1977; **95**: 2559–64.

[18]Drucker MM, Blondheim SH, Wislicki L. Factors affecting acetylation in vivo of para-aminobenzoic acid by human subjects. *Clin Sci* 1964; **27**: 133–41.