Separation of *p*-Aminobenzoic Acid Derivatives by Paper Chromatography

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According to the literature, *p*-aminobenzoic acid (PAB) is excreted in the urine mostly as the free acid and its *N*-acetyl derivative; the remaining part is eliminated conjugated with glucuronic acid and as *p*-aminohippuric acid (PAH) (Ellinger & Hensel, 1914; Hensel, 1914; Cohen & McGilvery, 1946; Salassa, Bollman & Dry, 1948). There is no agreement concerning the formation of PAH. Thus, Beyer, Mattis, Patch & Russo (1945) attach little importance to PAH formation, and according to Quick (1926, 1932 *a, b*) PAH formation does not even occur. The technical difficulties encountered on attempting simultaneous demonstration of PAH and PAB probably explain these divergent results, as the methods at present in general use are based solely upon the reactions of the aromatic amino group.

It is important for a number of reasons to have available a method permitting simultaneous determination of PAH and PAB. Thus, Schreiner, Wesson & Anslow (1949) reported that one of the widely used PAH preparations was contaminated with 23% of PAB. Lundquist (1945) and Halmágyi & Kelemen (1949) reported that the renal clearance of PAB, provided that the level in the plasma is low and urine flow is high, yields values close to renal blood flow. The question arose whether these results were to be attributed to PAB itself or to its possible metabolic conversion to PAH. The methods used were in each case incapable of deciding this question. Furthermore, the simultaneous determination may be of some importance in investigation of the role of PAB and *p*-aminosalicylic acid (PAS) in the metabolism of *Mycobacterium tuberculosis* (Landy, Larkum & Oswald, 1943; Bird, 1947; Lutz, 1949). Numerous other problems, which need not be enumerated here, could be attacked, using a method of this kind.

Ekman (1948) chromatographed a number of primary aromatic amines on filter paper with various organic solvents, using diazotization and *N*-ethyl-1-naphthylamine hydrochloride for colouring. Tabone, Robert & Troestler (1948) and Tabone & Robert (1949) separated *o*-, *m*- and *p*-aminobenzoic acids and kynurenine by paper chromatography using a method similar to our own, but found *n*-butanol inconvenient for their purpose. In what follows we describe the use of filter-paper chromatography for attacking problems such as those outlined above.

METHODS

The determination was carried out by means of paper-partition chromatography, based on Ma & Fontaine's (1949) modification of the upward-running method of Williams & Kirby (1948). Urine (2.5–20 μ l.) was applied directly to the paper without any preliminary treatment. The solvent was *n*-butanol saturated with water, and the colouring agent was Ehrlich reagent (2 g. *p*-dimethylaminobenzaldehyde in 100 ml. 20% (w/v) aqueous HCl). Ochre-yellow spots developed suddenly on spraying, even at room temperature. Results must be read before the paper dries, that is within about an hour. The R_F values observed are shown in Table 1. The normal variations in pH and salt concentration of urines caused no alteration in the R_F values observed. The sensitivity of the reaction permits detection of as little as 1 μ g. of each of the three compounds. With increasing amounts the size of spot increases, and excessive amounts (40–60 μ g. on a 20 cm. strip) can cause the confluence of the spots. Therefore amounts in excess of 20 μ g. should be avoided by previously diluting the urine; 3.5–7.5 μ g. gave the best results. Even large excesses of two of the substances do not interfere with the determination of the third.

Table 1. R_F values of *p*-amino compounds by ascending paper chromatography, using filter paper Whatman no. 1 at room temperature in *n*-butanol-water

	R_F
<i>p</i> -Aminobenzoic acid (PAB)	0.68–0.72
<i>p</i> -Aminohippuric acid (PAH)	0.07–0.10
<i>p</i> -Aminosalicylic acid (PAS)	0.26–0.30

Using parallel strips with less than 20 μ g. of the substance being determined, measurement of the area of spot can be used for quantitative comparisons, the error being ± 5 –12%. Elution of unsprayed areas followed by photometric determination (also using Schiff base formation with *p*-dimethylaminobenzaldehyde) failed to give better results.

RESULTS AND DISCUSSION

On administering PAB orally or intravenously to healthy persons, resulting plasma levels of PAB lay in the range of 1.5–24 mg./100 ml. On examining the urine of these subjects with our method, three spots developed on the chromatograms. One of these spots corresponded to PAB, the second to PAH and the third, which was present even in the urine of persons not receiving PAB, was lemon coloured (not ochre yellow) and was identified by

us as urea (R_f , 0.25–0.31). We further observed that, on chromatographing with water-saturated isobutanol or phenol, urea separates well from PAS. The R_f values in isobutanol were: PAS, 0.40–0.45; urea, 0.20–0.25. In phenol values were: PAS, 0.57–0.60; urea, 0.65–0.70.

At plasma levels around 2 mg. total PAB/100 ml. the spot of PAB mostly failed to appear in the urine, the chromatogram indicating excretion predominantly of PAH. This seems to be an important fact concerning PAB clearance. The spot of PAB always appears at more elevated PAB serum levels, but the spot of PAH remains or even increases markedly in intensity. We eluted unsprayed PAH areas of our chromatograms and subjected the eluates to hydrolysis with mineral acid. The presence of glycine in the hydrolysate was then demonstrated on a second chromatogram sprayed with ninhydrin. Following mild hydrolysis of the urine with hydrochloric acid

the spot corresponding to PAB also becomes stronger and brighter. This may be due in part to hydrolysis of the *N*-acetyl derivative, but the phenomenon was not investigated in detail.

SUMMARY

1. A method using partition chromatography with filter paper permitted separation and determination of microgram quantities of *p*-aminobenzoic, *p*-aminohippuric, and *p*-aminosalicylic acids when all occurred together.

2. The method was used for investigating in what form *p*-aminobenzoic acid is excreted in humans. At all plasma levels investigated (up to 24 mg./100 ml.) *p*-aminohippuric acid was present in the urine.

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The Role of Glutamic Acid in the Transport of Potassium in Brain and Retina*

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In a previous paper from this laboratory (Stern, Eggleston, Hems & Krebs, 1949) it was shown that sliced brain tissue, when suspended in a saline medium containing glutamic acid, absorbs this amino-acid against a concentration gradient. Tissue slices thus afford a system in which problems of permeability and active transport can be studied under controlled conditions. The present paper is concerned with an *in vitro* study of the mechanism maintaining the high potassium gradient between nervous tissue and body fluids. The main result is

the demonstration that, apart from a source of energy such as glucose or lactate, L-glutamate is required for the maintenance of the high potassium concentration in brain and retina.

EXPERIMENTAL

Material. Guinea pig and rabbit brain were treated as described by Stern *et al.* (1949). Guinea pig kidney cortex and rat testis were handled in the same manner.

Ox eyes were removed at the abattoir immediately after the death of the animal, placed in ice water and carried to the laboratory. The eye-ball was opened by a circular cut

* Preliminary communication: Krebs & Eggleston (1949).