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A Chromatographic Method for the Estimation of Nicotinuric Acid and Nicotinamide

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The published methods for the estimation of nicotinuric acid in mixtures containing nicotinic acid and other related compounds depend either upon the colorimetric estimation of 'total nicotinic acid' before and after hydrolysis, different methods of hydrolysis being employed to convert nicotinuric acid and other derivatives into nicotinic acid (for example, Ellinger & Abdel Kader, 1949), or upon the microbiological assay of nicotinic acid with two micro-organisms, one of which also responds to nicotinuric acid whereas the other does not (Johnson, 1945). Since they measure nicotinuric acid by difference, these methods are not able accurately to estimate small amounts of this substance in the presence of a large excess of nicotinic acid, and consequently were unsuitable for use in the experiments described in the two previous papers (Jones & Elliott, 1959; Jones, 1959).

This paper describes a direct method for nicotinuric acid estimation, in which nicotinuric acid is first separated from interfering substances by paper chromatography and then estimated by measurement of its u.v. absorption after elution from the paper. This method has also been successfully adapted to the estimation of nicotinamide in the presence of nicotinic acid.

EXPERIMENTAL

Materials and general methods have been described by Jones & Elliott (1959).

Estimation of nicotinuric acid. Samples containing 5–100 μ g. of nicotinuric acid were spotted on to Whatman no. 3 paper to produce spots of 1 cm. diameter. The chromatograms were then developed in descending butan-1-ol saturated with water, for 18–48 hr., the solvent being allowed to drip off the bottom of the papers. After solvent development, chromatograms were dried for 30 min. at 50–60°. The spots of nicotinuric acid were located by making a contact print of the chromatogram by the method of Markham & Smith (1949) with unfiltered light from a mercury lamp. By making a tracing of the photograph and transferring this on to the chromatogram, the positions of the spots could be fixed exactly.

The spots of nicotinuric acid were cut out, each with a border the size of which was then adjusted so that all the pieces were of equal weight. It was generally possible to include the whole of the nicotinuric acid spot in 200 mg. of paper (about 10 cm.²). The pieces of paper were cut into strips 1–2 mm. wide and put in test tubes. A volume (5 ml.) of N-HCl was then added to each tube and they were left to stand with occasional shaking for 3 hr., in which period elution was found to be complete. After 3 hr. the fluid was poured off, centrifuged to remove small shreds of paper and its extinction was measured at 262 m μ against N-HCl. Simultaneously a piece of blank paper was cut from the band having the same R_F as nicotinuric acid and eluted exactly as described above. The extinction of this eluate constituted the 'paper blank' which was deducted from the experimental values. The amount of nicotinuric acid was calculated from a calibration curve separately constructed as described below.

Estimation of nicotinamide. Nicotinamide was estimated by the method described above for nicotinuric acid estimation except that chromatograms were developed for only

12 hr., and the extinctions of the final eluates were read at 261 $m\mu$, which is the wavelength of maximum absorption for nicotinamide in *N*-HCl. Since nicotinamide had a high R_F in butan-1-ol-water and its running was not greatly affected by the presence of salts, an adequate separation from other u.v.-absorbing substances was obtained with the shorter running time.

Notes on the estimation of nicotinuric acid

Chromatography. The chromatographic separation was markedly influenced by the amount of other substance in the samples. With the incubation mixtures used in the two previous papers (Jones & Elliott, 1959; Jones, 1959) satisfactory separations were obtained in 24–30 hr. with 0.2–0.6 ml. samples. If the incubation mixtures contained appreciable quantities of sucrose, however, chromatography had to be continued for 40–48 hr.

The method of locating the nicotinuric acid spots was found to be entirely satisfactory. If a spot, located as described, was cut from a chromatogram together with a border 1 mm. wide all round and the remainder of the chromatogram was then treated with cyanogen bromide and *p*-aminobenzoic acid, no nicotinuric acid could be detected on the surrounding paper.

Spectrum. The absorption spectrum of synthetic nicotinuric acid was measured and compared with that of nicotinic acid between 210 and 290 $m\mu$, 0.1 mm solutions in water (Fig. 1) and in *N*-HCl (Fig. 2) being used. Nicotinuric acid has a higher

molecular-extinction coefficient than nicotinic acid in both water and *N*-HCl over the whole of the range recorded, though the position of the maximum at about 262 $m\mu$ is virtually the same in each. As with nicotinic acid, the peak in the nicotinuric acid spectrum at 262 $m\mu$ is much sharper in *N*-HCl than in water and the molecular-extinction coefficient at this wavelength is also higher, so that *N*-HCl was chosen for the elution of nicotinuric acid from the chromatograms.

A sample of synthetic nicotinuric acid which had been chromatographed in butan-1-ol-water and eluted with *N*-HCl was found, when measured against a paper blank, to have the same absorption maximum as nicotinuric acid in pure solution in *N*-HCl. Variation in the strength of the HCl in the range from 0.9 to 1.1*N* led to no change in the extinction given by known amounts of nicotinuric acid or in the position of the absorption maximum.

Paper blank. The practicability of this method depended upon the amount of u.v.-absorbing material eluted from the paper itself ('paper blank'), being small and constant for different parts of a single sheet of paper. To examine these points, blank sheets of Whatman no. 3 paper were chromatographed in butan-1-ol-water for 24 hr. and dried. Several samples of equal weight were cut from the band in which nicotinuric acid would have run, were eluted as described above and the extinctions of the eluates measured at 262 $m\mu$ against *N*-HCl.

Fig. 1. Spectra of nicotinuric acid and nicotinic acid in water. Readings were taken at 1 $m\mu$ intervals (0.5 $m\mu$ intervals in the region of the peaks) with 0.1 mm solutions. —, Nicotinuric acid, $\lambda_{\max}$. 213 and 262 $m\mu$ (ϵ 8240 and 4310 respectively); - - -, nicotinic acid, $\lambda_{\max}$. 262 $m\mu$ (ϵ 3840).

Fig. 2. Spectra of nicotinuric acid and nicotinic acid in *N*-HCl. Readings were taken at 1 $m\mu$ intervals (0.5 $m\mu$ intervals in the region of the peaks) with 0.1 mm solutions. —, Nicotinuric acid, $\lambda_{\max}$. 210.5 and 262 $m\mu$ (ϵ 7440 and 6000 respectively); - - -, nicotinic acid, $\lambda_{\max}$. 211.5 and 261 $m\mu$ (ϵ 5200 and 5000 respectively).

The extinctions obtained with different samples taken from a single sheet of paper never varied by more than ± 0.006 , which corresponds to less than $\pm 1 \mu\text{g.}$ of nicotinuric acid. Although the paper blank was for practical purposes constant for a single chromatogram, it did vary somewhat from one chromatogram to another, generally falling in the range 0.04–0.08/200 mg. of paper. The main factors determining the magnitude of the paper blank seemed to be the time and the temperature at which the chromatogram was dried. It was not significantly reduced by washing the paper with 2*N*-acetic acid or 0.5 % disodium ethylenediaminetetraacetate before chromatography. Since $1 \mu\text{g.}$ of nicotinuric acid corresponds to an extinction of about 0.007, it is evident that neither the absolute size nor the variation of the paper blank precludes the possibility of estimating accurately quantities of nicotinuric acid of the order of a few micrograms.

'Zero time' and 'no nicotinic acid' blanks. The possibility of interference with the estimations by the presence in the incubation mixtures of u.v.-absorbing substances other than nicotinuric acid but having similar R_f values was examined in two ways. First, the u.v.-absorbing substances running in the nicotinuric acid area were estimated in an incubation mixture stopped at zero time ('zero time' blank). The mixture had the following composition, which is essentially the same as that used in experiments on nicotinuric acid synthesis (Jones & Elliott, 1959): 0.3 ml. of 0.1 *M*-phosphate buffer, pH 7.35; 0.03 ml. of 0.1 *M*- MgCl_2 ; 0.3 ml. of 0.1 *M*-potassium fumarate; 0.15 ml. of 0.01 *M*-adenosine triphosphate (ATP); 0.2 ml. of 0.308 *M*-glycine; 0.1 ml. of 0.154 *M*-potassium nicotinate; 1.0 ml. of 0.154 *M*-KCl; 1.0 ml. of tissue preparation; final volume, 3.08 ml. It was deproteinized without incubation and a sample chromatographed. The area where nicotinuric acid would have been expected to run and a paper blank were cut out and eluted. The extinction obtained for the 'zero time' blank agreed with the paper blank within the experimental error in several trials.

Secondly, experiments were carried out with mixtures incubated without nicotinic acid ('no nicotinic acid' blank). The mixtures were set up as above, except that they contained 0.154 *M*-KCl in place of potassium nicotinate, and were incubated for 2 hr. at 38°. After incubation, but before deproteinization, appropriate volumes of 0.154 *M*-potassium nicotinate and water were added. Samples were chromatographed and the nicotinuric acid area was eluted. The extinctions obtained for the 'no nicotinic acid' blank agreed with the paper blank over a wide range of nicotinic acid concentrations.

These experiments showed that no u.v.-absorbing

material interfering with nicotinuric acid estimation (and incidentally no nicotinuric acid) was present in the incubation mixtures either initially or after incubation without nicotinic acid. It was similarly shown that the components of the incubation mixtures used in experiments with the soluble enzyme preparation (Jones, 1959), especially co-enzyme A and hydroxylamine, did not interfere with the estimation of nicotinuric acid by this method. It was therefore assumed that, apart from the paper blank, all u.v.-absorbing material eluted by the method described was nicotinuric acid. In experimental determinations both a paper blank and either a 'zero time' or a 'no nicotinic acid' blank were determined on each chromatogram. If the extinctions differed by more than 0.01 the chromatogram was discarded.

Calibration curves. With this method, linear calibration curves were obtained over the range 0–200 $\mu\text{g.}$ of nicotinuric acid with synthetic nicotinuric acid, both alone and when mixed with nicotinic acid. In order to obtain a calibration curve under experimental conditions incubation mixtures were set up as described above, except that they contained 0.154 *M*-KCl in place of potassium nicotinate. After incubation for 2 hr. at 38°, but before deproteinization, 0.1 ml. of 0.154 *M*-potassium nicotinate and appropriate amounts of nicotinuric acid and water were added. The mixtures were immediately deproteinized and the nicotinuric acid was estimated as described. In this way a calibration curve was obtained which was

Fig. 3. Calibration of the method of estimation of nicotinuric acid and nicotinamide described in the text. $\circ$, Nicotinuric acid; $\bullet$, nicotinamide. The amounts of nicotinuric acid and nicotinamide given refer to the amounts chromatographed.

linear over the range 0–100 $\mu\text{g.}$ of nicotinuric acid on the paper (Fig. 3). Calculation of the slope of the straight line gave a value of 0.00707 for the extinction per $\mu\text{g.}$ of nicotinuric acid (1.273 per $\mu\text{mole.}$). This value was used to calculate the amounts of nicotinuric acid from observed extinctions. A calibration curve or a standard amount of nicotinuric acid was not normally run with each experiment since the agreement between separately determined calibration curves was very good. Occasional determinations of known amounts of nicotinuric acid showed excellent agreement with the expected values.

Notes on the estimation of nicotinamide

Blanks. Measurements of the paper blank in the nicotinamide band showed that the values were similar to those in the nicotinuric acid band and that their variation on a single sheet of paper was small, corresponding to not more than $\pm 1 \mu\text{g.}$ of nicotinamide.

Experiments similar to those described above were performed to determine the 'zero time' and 'no nicotinic acid' blanks in the nicotinamide area. It was found that the agreement between these blanks and the paper blank depended upon the tissue preparation used. For example, with rat-kidney mitochondria both the 'zero time' and the 'no nicotinic acid' blanks were the same within the experimental error as the paper blank; with rat-kidney homogenates the 'zero time' blank was the same as the paper blank, but significant amounts of u.v.-absorbing material appeared in incubation mixtures containing no nicotinic acid; and with rat-liver homogenates u.v.-absorbing substances were found in both types of blank. The amounts of u.v.-absorbing substances were in no case very large, corresponding to not more than 10–20 $\mu\text{g.}$ of nicotinamide in a 3 ml. incubation mixture. When samples of the blank incubation mixtures were chromatographed and treated with cyanogen bromide and *p*-aminobenzoic acid it was found that the relative amounts of nicotinamide present in the various mixtures corresponded, as accurately as this could be judged by visual comparison of the spots on the chromatograms, with the amounts found by elution and measurement at 261 $\text{m}\mu$. It seemed probable therefore that the u.v.-absorbing material found in the 'zero time' and 'no nicotinic acid' blanks was, in fact, nicotinamide, and for experimental purposes it was assumed that all u.v.-absorbing material found in excess of the paper blank was nicotinamide.

Calibration curves. With nicotinamide alone and with nicotinamide and nicotinic acid linear calibration curves were obtained over the range 0–50 $\mu\text{g.}$ of nicotinamide (higher amounts were not tested).

Calibration curves in which nicotinamide was added to the incubation mixtures were also shown to be linear over the range 0–40 $\mu\text{g.}$ of nicotinamide (Fig. 3). The calculated slope of this line gave a value of 0.00854 for the extinction per $\mu\text{g.}$ of nicotinamide (1.042 per $\mu\text{mole.}$).

DISCUSSION

The principal advantage of the method described for nicotinuric acid and nicotinamide estimation is that it is a direct method which is capable of estimating small quantities (0–100 $\mu\text{g.}$) of these substances in the presence of relatively large amounts of nicotinic acid and other related compounds, with an error of not more than $\pm 2 \mu\text{g.}$ These figures apply to the amounts of the substances chromatographed and some restriction is placed upon the sensitivity of the method by the rather small samples which have to be used. The method has proved satisfactory in studies of nicotinuric acid synthesis (Jones & Elliott, 1959; Jones, 1959), and in an unsuccessful search for an enzyme synthesizing nicotinamide from nicotinic acid and ammonia or other amino donors (Jones, 1958).

It must be emphasized that care is necessary in the application of the method. Since it depends upon the separation of nicotinuric acid or nicotinamide from other u.v.-absorbing substances before measurements of extinction are made, it is only specific in so far as the separation is complete. Consequently, in order that the results obtained in any particular case should be valid, it must be shown that such a separation has been achieved.

The chief disadvantage of the method is the long time required to complete an estimation, mainly in the period required for chromatography, but on the other hand it is possible to carry out a number of estimations simultaneously.

SUMMARY

1. A direct method for the estimation of nicotinuric acid and of nicotinamide in the presence of large amounts of nicotinic acid is described. It depends upon the separation of nicotinuric acid or nicotinamide from interfering substances by paper chromatography and the measurement of their u.v. absorption after elution from the paper.

2. Linear calibration curves have been obtained for nicotinuric acid over the range 0–100 $\mu\text{g.}$ and for nicotinamide over the range 0–40 $\mu\text{g.}$

3. The u.v.-absorption spectrum of nicotinuric acid in water and in *N*-hydrochloric acid has been measured.

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Synthesis of ^{35}S -Labelled Arylsulphates by Intact Animals and by Tissue Preparations, with Particular Reference to L-Tyrosine O-Sulphate

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The combination of sulphuric acid with phenolic compounds to form arylsulphates is well known to occur in the animal body and certain arylsulphates are present normally in the urine of higher animals. Recently a new arylsulphate, namely L-tyrosine O-sulphate, has been reported in the urine of normal and abnormal human subjects (Tallan, Bella, Stein & Moore, 1955). The possible occurrence of this compound in the urine of species other than man has been investigated in the present work.

The synthesis of arylsulphates by tissue preparations has been studied in some detail (e.g. Bernstein & McGilvery, 1952; DeMeio, Wizerkaniuk & Fabiani, 1953; Robbins & Lipmann, 1957; Segal, 1957). Attempts to synthesize L-tyrosine O-sulphate, however, from L-tyrosine by various tissue preparations have so far been unsuccessful (Segal, 1957; Dodgson, Rose & Tudball, 1959). Synthesis of this particular arylsulphate has been investigated in the present work with preparations of the liver and kidney tissue from several species. The technique employed was capable of detecting the synthesis of small amounts of arylsulphate and depended on the use of ^{35}S -labelled sulphate as a source of sulphate. The tissue preparations used were active in synthesizing several arylsulphates from phenolic substrates under differing experimental conditions. It was not possible, however, to demonstrate synthesis of L-tyrosine O-sulphate from L-tyrosine.

MATERIALS

The potassium salts of phenylsulphuric acid and of *p*-cresylsulphuric acid were prepared by the method of Feigenbaum & Neuberg (1941). The *p*-toluidine salts of the products were prepared (Barton & Young, 1943), and had satisfactory melting points.

L-Tyrosine O-sulphate. The monopotassium salt of this compound was prepared as described by Tallan *et al.* (1955) and was isolated as the dihydrate (Found: N, 4.1; S, 9.4; K, 11.8. Calc. for $\text{C}_9\text{H}_{10}\text{O}_6\text{NSK}\cdot 2\text{H}_2\text{O}$: N, 4.2; S, 9.6; K, 11.7%).

Potassium *p*-ethylphenylsulphate. Freshly distilled chlorosulphonic acid (1.6 ml., 2.9 g.) was added dropwise, and with shaking, to a solution containing 10 ml. of carbon disulphide and 10 ml. of dimethylaniline cooled in a bath containing chipped ice. Freshly distilled *p*-ethylphenol (3 g.) was added to this mixture, which was warmed to room temperature and shaken mechanically for 1 hr. This reaction mixture was added slowly, and with continuous stirring, to an ice-cold solution containing 7 g. of KOH in 10 ml. of water. The resulting mixture was centrifuged at 500 *g* for several minutes and the supernatant liquor was discarded. The precipitate was stirred with 10 ml. of ethanol and the mixture was centrifuged as before. The supernatant was discarded, the precipitate was transferred to a Buchner funnel and was dried by suction. The resulting pale-yellow solid was washed several times with ether and yielded 2.8 g. of material, which was dissolved in 10 ml. of hot water and was rapidly filtered. Large plates were obtained from the pale-yellow filtrate (Found: C, 40.1; H, 3.4; S, 13.3; K, 16.3. Calc. for $\text{C}_8\text{H}_9\text{O}_4\text{SK}$: C, 40.0; H, 3.8; S, 13.3; K, 16.3%).

Potassium *p*-acetylphenylsulphate. This was prepared as described by Dodgson & Spencer (1953) (Found: C, 37.9; H, 2.7; S, 12.4. Calc. for $\text{C}_8\text{H}_7\text{O}_5\text{SK}$: C, 37.8; H, 2.8; S, 12.6%).

L-Tyrosine methyl ester. This compound was prepared by the method of Fischer & Schrauth (1907). The product had m.p. 134–136°, $[\alpha]_D^{25} + 30^\circ$ in methanol (*c.* 1). Fischer & Schrauth give m.p. 135–136°, $[\alpha]_D^{20} + 26^\circ$ in methanol (Found: C, 61.1; H, 6.3; N, 7.2. Calc. for $\text{C}_{10}\text{H}_{13}\text{O}_3\text{N}$: C, 61.5; H, 6.7; N, 7.2%).

L-Tyrosine amide. This was prepared from L-tyrosine methyl ester by the method of Blau & Waley (1954). Colourless crystals were obtained, m.p. 152–153°: L-tyrosine amide has m.p. 153–154° (Found: C, 59.8; H, 6.9; N, 15.6. Calc. for $\text{C}_9\text{H}_{12}\text{O}_3\text{N}_2$: C, 59.9; H, 6.7; N, 15.5%).