now better known. The section on interpolation at the end of the introduction must be read in order to use the tables efficiently; the methods of interpolation with reference to which several of the tables have been arranged will be novel to most statisticians.

Professor Fisher sends the following erratum. The formula for the range at the top of p. 8 should be

$$1/2PQ = 2/(1-R^2).$$

HAROLD HOTELLING
COLUMBIA UNIVERSITY

SPECIAL ARTICLES

THE TOXICITY AND ABSORPTION OF 2-SULFANILAMIDOPYRIDINE AND ITS SOLUBLE SODIUM SALT[1]

THE discovery of the chemotherapeutic activity of 2-sulfanilamidopyridine in experimental pneumococcus as well as streptococcus infections in mice[2] has led to a trial of the drug in human pneumococcus infections, as well as in various other bacterial diseases. The clinical use of 2-sulfanilamidopyridine was undertaken before a chemical description or any adequate pharmacological and toxicological study of the drug was reported. The only study of the toxicity of the drug is that reported by Wien,[3] who concluded "that this substance has a big advantage over sulfanilamide in being much less toxic," having "about one fourth the toxicity of sulfanilamide."

The above conclusion of Wien has been widely quoted. However, since all Wien's toxicity data were obtained by oral administration of an acacia suspension of the drug and since 2-sulfanilamidopyridine is a rather insoluble substance and is poorly absorbed, considerable doubt exists as to the validity of his conclusion. It has been shown that many sulfanilamide derivatives of low solubility owe their lack of toxicity to poor absorption from the gastro-intestinal tract, and it has been pointed out that determinations of the toxicity of such compounds administered by the oral route may be misleading because of low absorption when large doses are given.[4, 5, 6] Our finding, that when 2-sulfanilamidopyridine is given as its very soluble sodium salt, both the toxicity and the absorption are quite different from that of 2-sulfanilamidopyridine itself, would appear to justify the publication of this preliminary note.

The sodium salt of 2-sulfanilamidopyridine[7] was prepared as follows. One part of sulfanilamidopyridine was suspended in 20 volumes of boiling 95 per cent. alcohol, and 1.5 moles of 1.3 M alcoholic sodium hydroxide were added per mole of sulfanilamidopyridine. The solution was chilled 2 hours in ice, and the white crystalline precipitate filtered, washed with cold alcohol and dried at 110°. Yield, 80 per cent. Titration with standard acid and methyl red showed 98.9 per cent. purity; colorimetric analysis[8] indicated 100.1 per cent. purity. These values were essentially unchanged by recrystallization from 95 per cent. alcohol. In this colorimetric analysis, it is important to note that unless the solution of the sodium salt is treated with the strong trichloroacetic acid solution before dilution, considerably lower (about 8–10 per cent.) results are obtained. On this account, further work was done with the sodium salt to establish its purity. One gram of sodium salt was titrated with standard acid to neutrality using methyl red, the precipitate was filtered off, washed with a small amount of cold water, and dried at 110° for a few minutes and drying completed in a vacuum desiccator. A 97.1 per cent. recovery of the sulfanilamidopyridine resulted, m. p. 190.4–190.9°, unchanged by admixture with a carefully purified sample of the original material. The sodium salt was prepared also by adding sufficient alcohol to completely dissolve the sulfanilamidopyridine, adding alcoholic sodium hydroxide to the solution and immediately cooling. Yield, 71 per cent. A third preparation of the sodium salt was made without the use of alcohol, by dissolving the sulfanilamidopyridine in 1.5 moles of warm 3 M aqueous sodium hydroxide and chilling. Yield, 60 per cent. (A further 20 per cent. yield may be obtained by adding 20 parts of absolute alcohol to the mother liquor). The sodium salts prepared by all three methods appeared to be identical. We have no explanation to offer for the low results which are obtained by the usual colorimetric method.

The sodium salt is a white product, crystallizing from 95 per cent. alcohol in clusters of radiating thin rods. It melts with decomposition at 316.5–317°. Its solubility in the non-aqueous solvents is low, as would be expected of an organic sodium salt. It dissolves in water to the extent of approximately 63 grams per 100 cc (25°). The pH of a 1 per cent. aqueous solution is 10.4; that of a 10 per cent. solution, 11.0.

Acetylsulfanilamidopyridine was prepared by treatment of a warm aqueous solution of the hydrochloride

[1] This investigation has been aided by a grant from The John and Mary R. Markle Foundation.

[2] Whitby, *Lancet*, 1: 1210, 1938.

[3] Wien, *Quart. Jour. Pharmacy and Pharmacology*, 11: 217, 1938.

[4] Marshall, Cutting and Emerson, *Jour. Am. Med. Assn.*, 110: 252, 1938.

[5] Marshall, Cutting and Cover, *Bulletin Johns Hopkins Hosp.*, 63: 318, 1938.

[6] Finestone, Bliss, Ott and Long, *Bulletin Johns Hopkins Hosp.*, 62: 565, 1938.

[7] The 2-sulfanilamidopyridine was kindly furnished by Merck and Company and by the Calco Chemical Company.

[8] Sulfanilamidopyridine can be estimated by the same procedure as used for sulfanilamide.[9, 10]

of sulfanilamidopyridine with acetic anhydride, followed by addition of sodium acetate. Recrystallized from 30 per cent. acetic acid, it melted at 225.6–226.3°. Its sodium salt was prepared in boiling alcoholic solution as described above and precipitated by the addition of 250 parts of ether.

A study was made in mice of the blood concentration-time curves of sulfanilamidopyridine administered orally either as an acacia suspension of the free acid or as a solution of the sodium salt. Blood concentrations were determined by the method previously described for sulfanilamide.[9, 10, 11] Doses of 0.4, 1.0, 3.0, 6.0 and 16.0 grams per kilogram of the acacia suspension yielded blood values of increasing amount, but these values were not at all proportional to the dose. The maximum blood concentrations attained with 6 and 16 grams were around 50 mgm per cent., but in some instances blood levels reached by 6 were greater than those with 16 grams. On the other hand, when the extremely soluble sodium salt was given, it was more readily absorbed, and blood levels attained with doses of 0.4, 1.0 and 2.0 grams[12] per kilogram were roughly proportional to the dose. The maximum blood levels obtained with 1 gram (40 mgm per cent.) were nearly as high as with 16 grams of the suspension, while 2.0 grams gave blood levels of 65 mgm per cent. or over—higher than those obtained with eight times this amount in the form of a suspension of the difficultly soluble acid itself.

When a solution of sodium sulfanilamidopyridine is given to mice *per os,* the toxicity of the substance is greater than that of sulfanilamide. Thus, all in groups of ten mice given 3.0 and 4.0 grams per kilogram died, 60 mice given 2.0 grams gave 50 per cent. mortality, and ten mice given 1.0 gram had severe symptoms but all survived. The symptoms caused by this drug were quite different from those seen after toxic doses of sulfanilamide in that more stimulation and less depression of the nervous system occurred. A rigidity of the tail (the Straub morphine reaction) appeared first, then violent excitement with ataxia, dyspnoea and tetanic convulsions followed by a mixed type occurred. These symptoms appeared in one-quarter hour, and death occurred in less than 4 hours. The blood levels in mice at death have varied from 70 to 94 mgm per cent. (average 83). The administration of an amount of sodium carbonate solution equivalent in base to 4.0 grams per kilogram of sodium sulfanilamidopyridine to mice caused no symptoms. Wien has given the $L D_{50}$ for mice as 16.6 grams per kilogram, a figure about 8 times as great as our value found by using the sodium salt. We have attempted to check Wien's determination of the toxicity of an acacia suspension of the free acid, but due to erratic absorption with large doses and deaths at quite low blood levels, we concluded that the method was worthless and that death may not be the result of the real toxicity of the drug. However, we observed the same symptoms 1 to 3 hours after giving the acacia suspension of the free acid. Wien found that a dog given 1 gram per kilogram of the 2-sulfanilamidopyridine *per os* daily for seven days exhibited no symptoms. We gave a small dog on two occasions a single dose of 1.0 gram per kilogram of the sodium salt, and although a large part of the dose was vomited, the animal showed severe symptoms in the form of tonic and clonic convulsions with occasional opisthotonos. (The maximum blood concentration was 33 mgm per cent.) Another dog given 1.0 gram per kilogram subcutaneously exhibited the same severe symptoms and died with a blood level of 62 mgm per cent.

Blood concentration-time curves and the percentage excretion of the drug in the urine were studied in dogs to which 0.1 gram per kilogram was administered in different ways by mouth. When sulfanilamidopyridine was given without water in gelatin capsules or compressed tablets absorption was poor and erratic. The administration of water with the drug had no influence on absorption in the case of capsules, but did appear to increase absorption when tablets were used; also, when given suspended in acacia or water, absorption was more rapid. The greatest, most rapid and regular absorption was seen when the drug was given dissolved in hydrochloric acid or in sodium bicarbonate solution or as the soluble sodium salt.

These observations appear to indicate that four factors condition the absorption in dogs: (1) the solubility of the preparation, (2) the particle size, (3) the state of suspension, and (4) the rapidity of passage of the drug from stomach to intestine. Since in the case of patients given tablets of sulfanilamidopyridine absorption appears to be erratic,[13] various methods of administration are being tried on man in order to obtain more complete and constant absorption.

Sulfanilamidopyridine is apparently excreted unchanged in the urine of the dog, but in the rabbit and man it is excreted partly in the free and partly in a conjugated form. In the mouse, only a very small amount of the drug appears to be conjugated. One of us (B) has isolated the conjugated form from the urine of a patient receiving the drug and found it to be p-acetylaminobenzenesulfamidopyridine. Since this substance is even less soluble than the sulfanilamidopyridine and probably less readily absorbed, its sodium salt has been used for toxicity determination in mice.

[9] Marshall, *Jour. Biological Chemistry,* 122: 263, 1937.
[10] Marshall and Litchfield, SCIENCE, 88: 85, 1938.
[11] Marshall and Cutting, *Bulletin Johns Hopkins Hosp.,* 63: 328, 1938.
[12] All values for doses and blood concentrations are expressed in terms of sulfanilamidopyridine.
[13] Long: Personal communication.

Of ten mice given 4.0 grams per kilogram five died; the blood levels in two at death were found to be 77 and 106 mgm per cent. It appears that the acetyl-derivative of sulfanilamidopyridine is of the same order of toxicity as the unconjugated compound if blood concentrations are taken into consideration.

In conclusion, we wish to emphasize that sulfanilamidopyridine on the basis of blood concentration values appears to be more toxic than sulfanilamide. Until more is known about the drug, it should not be used in conditions where sulfanilamide has been shown to be effective.

E. K. MARSHALL, JR.
A. C. BRATTON
J. T. LITCHFIELD, JR.
DEPARTMENT OF PHARMACOLOGY
AND EXPERIMENTAL THERAPEUTICS,
THE JOHNS HOPKINS UNIVERSITY

THE APPLICATION OF THE NITROGEN ISOTOPE N^{15} FOR THE STUDY OF PROTEIN METABOLISM

THE production of nitrogen with an increased concentration of the isotope of atomic weight 15 (N^{15}) by Urey and his collaborators has opened the possibilities of investigating the metabolism of amino acids, proteins and other nitrogenous compounds in normal healthy animals. The principles underlying the procedure are similar to those which have been successfully employed in the investigation of the fate of fats, steroids and other compounds, with deuterium as a tracer. The substance to be investigated is synthesized in the laboratory in such a manner that one of its atoms contains an increased concentration of isotope, in the present case[1] by starting the synthesis with ammonia N^{15}. The rarity and value of the isotopic ammonia compelled the development of methods for amino acid synthesis which should lead to complete recovery of the isotope. The methods employed were modifications of the phthalimide synthesis of Gabriel and of the catalytic reduction of α-keto acids in the presence of ammonia according to Knoop. The following isotopic and racemic amino acids are now available for biological investigations, all of which contain nitrogen with more than 2 per cent. N^{15} as compared with the normal abundance of 0.368 per cent.: glycine, alanine, nor-leucine, tyrosine, phenylalanine, glutamic acid, aspartic acid, lysine and leucine. The latter compound also contains, besides the nitrogen isotope, stably (carbon) bound deuterium, and has been resolved in the laboratory into its optical isomers.

The biological application of such substances requires a highly sensitive micro method for the analysis of the N^{15} content in the nitrogen of organic compounds. The only practical procedure requires the use of a mass spectrometer. The nitrogen of the compound is converted to ammonia, elementary nitrogen is liberated from it in a high vacuum system and this admitted to the vacuum tube containing the mass spectrometer proper. In this tube the gas is ionized and under the influence of electric and magnetic fields is dispersed into a spectrum of the component masses. The instrument constructed in our laboratory requires less than 1 mg of nitrogen for an analysis and has a sensitivity of 0.003 per cent. N^{15} when normal nitrogen is analyzed. Any of the amino acids listed above can thus be mixed *in vivo* or *in vitro* with several hundred times its weight of the normal analogue before the analytical methods miss the isotope label.

The concentration of N^{15} in the nitrogen of ordinary casein as well as of ten different natural amino acids was determined and found to be the same as in the nitrogen of air, a finding which indicates that both isotopes of nitrogen are treated indiscriminately in anabolic and catabolic processes.

The nitrogen in organic linkage, as for example in amino acids, is stably bound and does not exchange with the nitrogen of other nitrogenous compounds with which it is brought in contact. This was established by the investigation of ten different systems, each of which contained one normal and one isotopic compound. Whenever, in experiments either *in vivo* or *in vitro,* compounds are observed which contain more than the normal abundance of isotopic nitrogen, the formation of such compounds must therefore be ascribed to chemical reactions.

An investigation on hippuric acid formation has already been reported[2]; other experiments on protein metabolism have now been carried out. The first is concerned with the much discussed question as to whether the animal organism can utilize the nitrogen of dietary ammonia for amino acid formation. Rats were given an ordinary stock diet to which were added benzoic acid and isotopic nitrogen as ammonium citrate. The experimental conditions under which the animals were kept were so chosen that most of the glycine excreted in the urine as hippuric acid was newly formed. This contained a small but significant amount of isotope, indicating that a small part of the glycine had been formed from ammonia nitrogen.

Another experiment carried out with immature rats was still more illuminating. The animals were given a protein-low diet to which isotopic ammonium citrate had been added. After five days they were killed, the carcasses were hydrolyzed and the following compounds were isolated in pure form; glycine, glutamic acid, aspartic acid, proline, histidine, lysine, arginine and creatine. All, with the exception of lysine, con-

[1] The authors are highly indebted to Professor H. C. Urey for the valuable gift of nitrogen isotope.

[2] R. Schoenheimer, D. Rittenberg, M. Fox, A. S. Keston and S. Ratner, *Jour. Am. Chem. Soc.,* 59: 1768, 1937.