and the mother liquor were found to contain oxalic acid. This was removed by dissolving in water, neutralizing, and adding a solution of calcium acetate. The calcium oxalate was filtered off and the oxalic acid isolated and positively identified by melting-point and mixed melting-point determination.

The filtrate from the calcium oxalate was re-acidified and extracted, yielding further crystals. The main crystalline product was recrystallized from ethyl acetate-petrol; melting-point thus raised to 186° (slight decomposition), colourless bipyramidal crystals, yield 4.1 g. (Found on sublimed sample C, 36.10; H, 3.07%. N, S, halogen, absent. Mol. wt. (Rast), 143, 135. Equivalent by titration 67.2. $C_4H_4O_6$ requires C, 36.38; H, 3.05%; mol. wt. 132; equivalent (dibasic), 66.0.) The acid was optically active, $[\alpha]_{5461}^{18} = -119.3^\circ$, $[\alpha]_{5790}^{18} = -105.1^\circ$ in 10% aqueous solution. It formed a sparingly soluble crystalline barium salt and had similar properties to the ethylene oxide- $\alpha\beta$ -dicarboxylic acid isolated by Sakaguchi *et al.* (1939) from the metabolic products of *Monilia formosa* n.sp. and *Penicillium viviferum* n.sp. This acid had $[\alpha]_D^{20} = -100.6^\circ$. Our melting-point is somewhat higher than that re-

corded by Sakaguchi *et al.* (1939) and by Kuhn & Zell (1937), which was 180°, but this may depend on the rate of heating.

For further confirmation our acid was converted into the *l*-rotatory chloromalic acid by means of HCl in ether. After crystallization from ether-light petroleum the product had m.p. 173° but showed definite signs of softening at 166°. Sakaguchi *et al.* and Kuhn & Zell give the melting-point of the chloro acid as 166°. (Found: Cl, 20.91%; $C_4H_5O_5Cl$ requires C, 21.04%.) The optical rotation was determined in ethyl acetate. Found: $[\alpha]_{5461}^{18} = -36.4^\circ$, $[\alpha]_{5790}^{18} = -31.6^\circ$ ($c = 0.92\%$ by wt.). Sakaguchi *et al.* record the rotation as $[\alpha]_D^{25} = -31.5^\circ$ in ethyl acetate.

SUMMARY

1. The metabolic products of an authentic strain of *Aspergillus fumigatus* Fresenius, grown on Czapek-Dox glucose solution, were investigated.

2. The antibiotic, helvolic acid, was isolated, together with considerable amounts of ethylene oxide- $\alpha\beta$ -dicarboxylic acid and small amounts of oxalic acid.

REFERENCES

- Anslow, W. K. & Raistrick, H. (1938*a*). *Biochem. J.* **32**, 687.
 Anslow, W. K. & Raistrick, H. (1938*b*). *Biochem. J.* **32**, 2288.
 Birkinshaw, J. H. & Raistrick, H. (1931). *Philos. Trans. B*, **220**, 245.
 Chain, E., Florey, H. W., Jennings, M. A. & Williams, T. I. (1943). *Brit. J. exp. Path.* **24**, 108.
 Crowfoot, D. M. & Low, B. W. (1943). *Brit. J. exp. Path.* **24**, 120.
 Dutcher, J. D. (1941). *J. Bact.* **42**, 815.
 Kuhn, R. & Zell, R. (1937). *Ber. dtsch. chem. Ges.* **59**, 2514.
 Menzel, A. E. O., Wintersteiner, O. & Hoogerheide, J. C. (1944). *J. biol. Chem.* **152**, 419.
 Oxford, A. E. & Raistrick, H. (1942). *Chem. Ind.* **61**, 128.
 Sakaguchi, K., Inoue, T. & Tada, S. (1939). *Zbl. Bakt., Abt. II*, **100**, 302.
 Waksman, S. A., Horning, E. S. & Spencer, E. L. (1943). *J. Bact.* **45**, 233.
 Weindling, R. (1937). *Phytopathology*, **27**, 1175.
 Weindling, R. & Emerson, O. H. (1936). *Phytopathology*, **26**, 1068.

The Quantitative Estimation in Urine of a Base Closely Allied to *l*-Nicotine, and the Absence of Alteration in Output when the Diet is Changed

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In a previous paper (Lockett, 1944*b*), a base was isolated from human urine, both of smokers and non-smokers, and was identified in final form as *l*-nicotine. Evidence was presented that the original unstable form of the base differed from *l*-nicotine. A similar base was detected in the urine of dogs;

solutions of this base and of *l*-nicotine, both as the hydrochlorides, which gave identical ultra-violet absorption spectra, were found to have identical pressor activity when injected intravenously into a cat under chloralose anaesthesia. No evidence as to the physiological significance of this base has yet

been obtained. A search for it in 17 tissues proved negative, but the quantities of material worked up were such that only a large store of the base could have been detected. It has already been shown (Lockett, 1944*b*) that the base is excreted by dogs that are receiving none of this base in the diet: it must therefore be synthesized by the animal, but this does not rule out the possibility that the amount excreted might be dependent upon some precursor in the diet. Such an explanation must, however, be considered improbable, for the accumulation of animal metabolic products is necessarily normally non-toxic in its extent, and a base having an activity at least as great as one-fifth that of adrenaline is far from inert. It was therefore decided as a preliminary step to discover whether the amount of the base excreted in the urine was altered by a drastic change in diet. For this purpose, a quantitative method for the estimation of the base in urine was worked out, and dogs were selected as subjects for the experiment on controlled and differing diets, the advantages being first that no exogenous source of nicotine would occur from unauthorized smoking, and second, that the diet and collection of urine could be completely supervised. The dogs were under the care of Dr E. B. Verney.

METHODS

Ten bitches took part in the experiment, which lasted 15 days. On the first day the biscuit diet was begun; on the fourth day the dogs were put into metabolism cages, and thereafter the urine for the preceding 24 hr. was collected. When necessary the dogs were catheterized in order that a quantitative collection of urine should be made. Each day the dogs, after they had urinated, were put out on the roof under supervision whilst their cages were

cleaned, and were immediately returned to their cages. The dogs were taken out of their cages and were fed the prescribed diet one at a time, under supervision, between 3.30 and 4.30 p.m. Immediately each had finished its meal, it was returned to its cage. The biscuits used were those described previously (Lockett, 1944*b*). These biscuits (104 oz.) were found to contain none of the base A. A total of 82 oz. of the biscuits was fed to the dogs on each of the first 7 days; from the eighth to the fifteenth day, a total of 63 oz. of meat was fed daily. The meat used for the whole period of this experiment was minced, mixed well, and 2 lb. were used for control extractions; the rest was stored in the ice-chest, and was fed according to plan.

The quantitative extraction of the base from urine. Earlier work (Lockett, 1944*a, b*) showed that the base A was not precipitated by basic lead acetate solution; was adsorbed by blood charcoal from which it could be eluted by dilute acid; and was very readily extracted by ether or by chloroform from alkaline solution, passing with great rapidity from such extracts to dilute aqueous acid. Obviously the fewer manipulations required, the less the danger of loss of the base during quantitative extraction. The aim was to obtain a solution of the base suitable for its quantitative estimation by means of its absorption in the ultra-violet. The method finally adopted is described in scheme 1, as it was used for the dog urine. The mixed urine from the 10 dogs for the preceding 24 hr., of total volume varying from 1.5 to 2.0 l., and of sp.gr. 1.033–1.042, was filtered through muslin and was then worked according to scheme 1. Quantitative estimations of the output of the base were made on the urine obtained on each of the last 4 days of the biscuit diet, the first 4 days, and the last day of the meat diet.

Treatment of the control solutions. The control solutions were designed to show whether or not loss of the base had occurred at any stage during its quantitative extraction from the urine. A final repetition was made of each stage as a control, and the resulting solutions were numbered according to the stage they controlled (see scheme 1). The control solutions derived from the last 4 days of the biscuit

Scheme 1. *Quantitative extraction of the base from urine*

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Urine, approximately 2 l., precipitated by basic lead acetate
↓
Filtrate, obtained by suction, 24 hr. after precipitation, freed of lead by H2S
↓
Filtrate concentrated to approximately 300 ml.; neutralized with NaOH, made alkaline with 4 ml. of 10% NaOH/100 ml.
  of concentrate
↓
Extracted four times with 50 ml. of chloroform, shaking 15 min. each time. A fifth extraction, control 1
↓
First four chloroform extracts combined, shaken three times for 5 min. with 15 ml. of 1.5% HCl; fourth time, control 2
↓
Aqueous phases combined, chloroform gently boiled out; cooled, neutralized with NaOH
↓
Three treatments with 0.3 g. blood-charcoal, rocking for 20 min. Fourth treatment control 3, eluted at once
↓
First 0.9 g. blood-charcoal three times eluted with 15 ml. of 0.6% HCl, boiling and stirring. A fourth eluate, control 4
↓
First three eluates combined, cooled, neutralized with NaOH; made alkaline with 3 ml. of 10% NaOH; let stand 15 min.;
  filtered
↓
Extracted three times with 10 ml. ether. Fourth extraction, control 5
↓
First three ether extracts together shaken with 3 ml. of 1.2% HCl, twice, for 3 min. Third treatment, control 6
↓
Ether boiled out of the combined acid aqueous phases (6 ml.) in tubes in a water-bath at 90°. Volume brought to 6 ml.
  exactly

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Scheme 2. Treatment of control solutions

diet were combined under their respective numbers, B1 to B6. Similarly those from the first 4 days of the meat diet were combined as M1 to M6 respectively. The B and the M control series were worked up separately as shown in scheme 2.

The ultra-violet absorption spectra of the solutions. Control solutions B1 and B2, M1 and M2, B3 and B4, M3 and M4, B5 and B6, M5 and M6, respectively, were mixed. The ultra-violet absorption spectra of all these solutions, day 5 to day 8 of the biscuit diet, day 1 to day 4, and day 8 of the meat diet, and the control solutions were photographed.

Examination of the meat to determine whether it contained the base A. The 2 lb. of meat reserved for the diet control were three times extracted with water at 60–64° for 8 hr., straining and pressing out, and reserving the fluid each time. Finally, the meat was four times extracted with water for 4 hr. at 90°. The process was carried out continually, and the fluid extracts were stored throughout in the ice-chest. The final meat residue on re-stewing and being put through the mangle gave only a watery pale yellow fluid; this was added to the other extracts, total volume 4.6 l. This fluid collection was worked up by a process similar to that shown in scheme 1, the volume of the final solution being 6 ml.

RESULTS

The meat control solution showed absorption at 2600 Å. in the ultra-violet (Fig. 1). It was therefore decided to examine this solution for pressor activity in a cat, and if it were pressor, to equate its pressor action with a solution of *l*-nicotine hydrochloride, and subsequently to compare the ultra-violet absorption of the two bases in acid and alkaline solutions of such strength that the pressor activities were equivalent.

The meat control solution (5.1 ml.) was neutralized with NaOH, and was brought to 8.0 ml. with water. A solution of *l*-nicotine hydrochloride was prepared from the *d*-tartrate. These solutions were compared for pressor activity on a cat weighing 1.9 kg., and prepared as previously described

(Lockett, 1944a). The results are summarized in Table 1. From this table it is seen that there is a

Fig. 1. Ultra-violet absorption curves. Daily output of the base A by the 10 dogs: (a) biscuit diet, (b) meat diet.

Table 1. *Blood-pressure responses of a cat, under chloralose anaesthesia, to the intravenous injection of the control solution, and a solution of l-nicotine hydrochloride*

Solution	Vol. injected (ml.)	Blood pressure (base-line in mm. Hg)	Rise in blood pressure (mm. Hg)
<i>l</i> -Nicotine	1.0	134	16
	1.5	138	19
Control (meat extract)	2.0	144	5
	3.0	142	11
<i>l</i> -Nicotine	0.75	143	14
	0.6	144	13
0.9 % NaCl	3.0	144	0

Fig. 2. Ultra-violet absorption curves: (a) *l*-nicotine solution, (b) meat control solution.

graded response to the varied doses of *l*-nicotine although the pressor responses are small, and that

0.6 ml. of the *l*-nicotine solution was rather more active than 3 ml. of the meat control solution.

The solutions for comparison in the ultra-violet were prepared as follows: to 3 ml. of the meat control solution as prepared for injection 0.5 ml. of 3 % HCl was added; to 0.6 ml. of the *l*-nicotine hydrochloride solution, 2.4 ml. of 0.9 % NaCl and 0.5 ml. of 3 % HCl were added. After each of these solutions had been photographed, 3 drops of 40 % NaOH were added to each and the process repeated. The control solutions were correspondingly prepared. The ultra-violet absorption curves are shown in Fig. 2. The absorption of the *l*-nicotine acid solution is considerably less than that of the meat control solution at 2600 Å., but the curves are roughly of the same type. In alkaline solution, whereas the absorption at 2600 Å. is not reduced in the case of the meat control and the shape of the curve is greatly altered, in the case of *l*-nicotine the absorption at 2600 Å. is reduced, the curve being but little changed. The two bases concerned are evidently not identical: whether or not the pressor response obtained in the meat control solution is to be attributed to the base present which absorbs at 2600 Å. can only be determined by the isolation of the base. This has not yet been done.

The results show (Fig. 1) that the output of the base A remains very constant, and is independent of the change in diet from biscuits to raw meat. That this finding is not due to some factor limiting the amount of the base extracted by the method given is shown by the absence of any absorption by the control solutions.

DISCUSSION

The results of this experiment clearly indicate that the output of the base A in the urine of 10 dogs on a controlled diet for 15 days was unaffected by a change in diet from biscuits to raw meat, confirming also the previous finding that the base A is of endogenous origin.

SUMMARY

1. A method for the quantitative estimation in urine of a substance similar to *l*-nicotine, and designated base A, is described.

2. It was found that the output of the base A in the urine of 10 dogs kept on a controlled diet for 15 days was unaffected by a change in diet from biscuits to raw meat.

REFERENCES

Lockett, M. F. (1944a). *J. Physiol.* **103**, 68.

Lockett, M. F. (1944b). *J. Physiol.* **103**, 185.