15163

Variations of Some Constituents in the Urine of Rats Receiving *dl*-Serine and *dl*-Alanine.*

WILLIAM H. FISHMAN† AND CAMILLO ARTOM. (Introduced by Harold D. Green.)
From the Department of Biochemistry, Bowman Gray School of Medicine, Wake Forest College, Winston-Salem, N.C.

In continuation of our investigation on the toxic effects of *dl*-serine and on the mechanism of their production, a more extensive study has now been made of the chemical composition of the urine of rats receiving *dl*-serine by stomach tube. In this regard, we have been interested mainly in the presence of pathological constituents (proteins and sugar-like substances), in the excretion of the administered amino acid and in the variations of certain other compounds or groups of compounds (ammonia, ethanolamine, reducing substances, and bisulfite-binding substances) which may presumably be related to the metabolism or serine.

For purpose of comparison, similar experiments have been performed on rats receiving *dl*-alanine ($CH_3 \cdot CHNH_2 \cdot COOH$), instead of *dl*-serine ($CH_2OH \cdot CHNH_2 \cdot COOH$).

Experimental. In the present experiments, groups of 3 or 4 albino rats, weighing about 100 g, were transferred from the stock diet to diet 4.[1] After 7 days, *dl*-serine (100 mg) was administered once daily to groups *1a* and *1b* and *3* by stomach tube for 12 consecutive days.‡ The animals were then maintained on the experimental diet without serine for 5 more days. In Groups *2a* and *2b*, the administration of *dl*-alanine (100 mg) was substituted for *dl*-serine; otherwise the experimental conditions were identical with those described above. All animals throughout the course of the experiment received a daily injection of a mixture of pure B vitamins,[2] either complete (Group *3*) or without pyridoxine (Groups *1a* and *1b*; *2a* and *2b*). Records were kept daily of body weight and food consumption.

The rats (1 or 2 animals per cage) were housed in metabolism cages supported by large glass funnels. In order to collect the urine separately from the feces, these were trapped by a 16-mesh galvanized screen wire in about the middle of the funnel: in addition, glass wool was inserted in the funnel stem. The urine specimens collected under toluene, were removed twice daily and stored in the refrigerator. The 24-hour collections from the rats of each group were pooled. Many of the analyses were performed on these pooled 24-hour collections (especially in the first days of amino acid administration.) Other analyses were done instead on aliquots of 2- to 4-day combined urines.

Methods. The urines were tested qualitatively for sugar-like substances (Benedict's) and for protein. When the urine contained protein, its approximate amount was estimated with the Esbach albuminometer.

"Total amino acids" were evaluated by a formol titration adapted to micro scale, after removal of the proteins, phosphates and carbonates. A description of the method adopted follows:

To 10 cc of urine, diluted to 50 cc, 2 g of permutit are added and the mixture shaken continuously for 5 minutes. The liquid is decanted and the treatment repeated twice. The final mixture is filtered. To a measured amount of the filtrate (*e.g.*, 35 cc) 1 cc of 5% colloidal ferric hydroxide and 3 cc of a 10% $BaCl_2$ solu-

* Aided by a grant from the John and Mary R. Markle Foundation. Preliminary report appeared in Abstracts of Meeting of Am. Chem. Soc., Sept., 1945.

† Present address: Departments of Surgery and Biochemistry, University of Chicago, Chicago, Ill.

1 Artom, C., and Fishman, W. H., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 239.

‡ One death occurred on the 3rd day (Group 3), and another on the 8th day (Group 1a) of serine administration.

2 Fishman, W. H., and Artom, C., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 241.

tion are added. After a short interval, the mixture is alkalinized with an excess of a 3% $Ba(OH)_2$ solution, brought to volume (*e.g.*, 50 cc) and filtered. To an aliquot of the filtrate (*e.g.*, 20 cc) 0.5 cc of a 1% solution of phenolphthalein in 50% alcohol is added, the solution acidified with 0.1 N HCl and the reaction made barely acid to phenolphthalein (approx. pH = 8) with 0.01 N solutions of NaOH and HCl. Fifteen cc of an 18% formol solution (approximately neutral to phenolphthalein) are now added and the mixture titrated with 0.01 N NaOH to a distinct red color (approx. pH = 9.0). A blank containing the same amounts of CO_2-free water, formol, and phenolphthalein is titrated. The volume is equalized by adding the required amount of CO_2-free water and the color of the unknown and blank solutions adjusted to the same tint§ with the standard 0.01 N solutions. The value of the blank is subtracted from the unknown and the results are expressed as micromoles of amino acid excreted per rat per day. Employing the technic as reported here, recoveries of 95 to 100% of added serine and alanine were obtained.‖

"Ethanolamine and unchanged serine" were determined according to a procedure described previously for mixtures of the pure substances in aqueous solution,[3] except that ethanolamine was adsorbed together with ammonia on permutit as described above. The amounts of urine employed were 0.5 to 2.0 cc for the determination of total serine plus ethanolamine and 5 to 10 cc of the permutit filtrate for the determination of serine. When the steam distillation of NH_3, as used in the method, is applied to the urine (or even to the permutit filtrate) in the absence of periodate, appreciable amounts of NH_3 arise from other substances. It is therefore necessary to perform a blank determination in the absence of periodate with every analysis whether on the urine or on the permutit filtrate. The data are expressed as micromoles of serine or ethanolamine excreted per rat per day. Because of the extent of the required corrections, the accuracy of the method as applied to the urine is certainly less than with solutions of the pure substances. However, in testing the technic, 90 to 100% of the ethanolamine and serine added (separately or together) to the urine were recovered.¶

Ammonia was determined in one of two ways. Where analyses were carried out for serine and ethanolamine (Groups *1a*, *1b*, and *3*), steam distillation was employed as indicated above. The ammonia artificially formed during the 6-minute period of distillation was evaluated by continuing the process for a second 6-minute period, and the value so obtained was used in correcting the ammonia values of the first 6-minute period. In Groups *2a* and *2b*, a formol titration was carried out according to the procedure described above, except that the treatment with permutit was omitted and the titration values were increased by a 3% correction. The difference between the values obtained with the formol titration before and after absorption was considered to represent the ammonia. When both ammonia procedures were applied to the same urine, satisfactory agreement was obtained. The values are expressed as micromoles of ammonia per rat per day.

Reducing substances. 1.0 cc of urine was diluted with H_2O to 10 cc and 1 cc of 30% NaCl solution and 1.0 cc of 5% colloidal ferric hydroxide were added. The mixture was brought to 15 cc with distilled H_2O and filtered after 5 minutes. 5.0 cc of the filtrate were made alkaline and the reducing power was determined according to the method of

§ In most cases, the final filtrates of the diluted urine after neutralization are practically colorless. Occasionally the use of a Walpole comparator was required to compensate for the slight residual color.

‖ Formol titration values always have a somewhat arbitrary significance. Our values probably include, in addition to the amino acids, lower polypeptides, and possibly amino bases, weakly dissociated, which might have escaped adsorption by permutit. Likewise, the possibility that traces of ammonia are also included cannot be ruled out completely.

3 Artom, C., *J. Biol. Chem.*, 1945, **157**, 585.

¶ Obviously, the serine figures may include other hydroxyamino acids, and the ethanolamine data, other non-volatile (relatively strong) bases with adjacent amino and hydroxy groups, which might have been adsorbed on permutit.

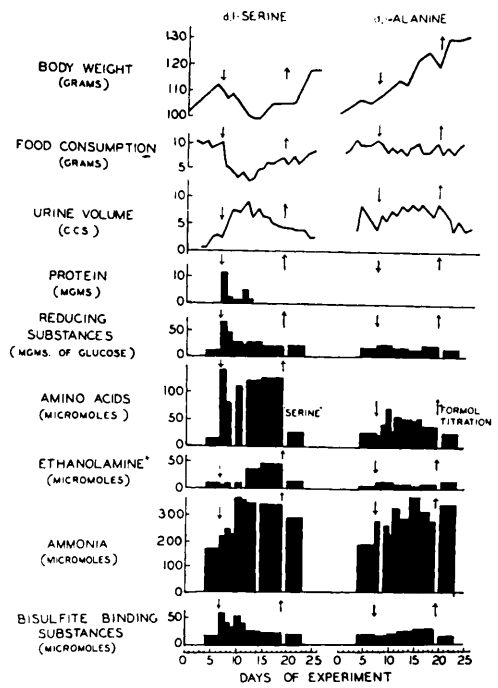

FIG. 1.

The variations in body weight, food consumption, volume and composition of the urine of rats to which either *dl*-serine or *dl*-alanine (100 mg per rat per day) was given by stomach tube. The arrows indicate the day on which the administration of the amino acid was started (↓), and that on which it was discontinued (↑). All rats were on an experimental diet (Diet 4) and received a daily injection of a mixture of pure B vitamins without pyridoxine.

Miller and Van Slyke.[4] The data are expressed as milligrams of glucose per rat per day.

Bisulfite-binding substances were determined on 2.5 cc of the urine which had been acidified with 1 cc of a 3% HCl solution and diluted with H_2O to 20 cc. The analyses were completed as described by Wortis, Bueding, and Wilson.[5] Two equivalents of I_2 are required for each free =CO or —CHO group. The results have been expressed accordingly as micromoles of carbonyl compound excreted per rat per day.

Results. In Fig. 1, data obtained from Groups *1a* and *1b*, receiving *dl*-serine, have been averaged and compared with similar data from Groups *2a* and *2b*, receiving *dl*-alanine.

In agreement with our previous results,[1,2] the administration of *dl*-serine causes a characteristic fall in food consumption and in body weight during the first week. This is followed by a partial recovery of growth and appetite in spite of the continued administration of the amino acid. In the case of the *dl*-alanine groups, there is no significant change in either the food consumption or the rate of growth upon administration of the amino acid.

As for the urinary findings in the animals of the serine group, some of the present results confirm and extend those of the preceding paper.[6] It should be remembered that the rats used in that investigation did not receive B vitamins.

The volume of urine eliminated after giving serine is probably increased with a maximum value at the fourth day, whereas little or no change was observed in the urine volume of the rats receiving *dl*-alanine.

Qualitative reactions for protein and sugar-like substances were positive in the first few days of serine administration and were negative in the alanine group. Evidence that glucose was present in the urine of the serine groups is supplied by the following findings: (1) Formation of CO_2 by baker's yeast. In the urine after fermentation, the Benedict's test became negative. (2) Formation of typical crystals of glucosazone in rather large amounts by reaction with phenylhydrazine. The osazone, recrystallized from alcohol, melted at 198°-199° (uncorrected). No depression of the melting point was observed upon admixture with an authentic sample of glucosazone.

As for the excretion of the amino acids, a substantial proportion (6-13%) of the serine administered is eliminated unchanged throughout the period of administration. On the other hand, on the basis of the formol titration values, much less of the *dl*-alanine administered (4% at most) is apparently eliminated as such. A significant increase in substances behaving as ethanolamine was detected only

[4] Miller, B. F., and Van Slyke, D. D., *J. Biol. Chem.*, 1936, **114**, 583.

[5] Wortis, H., Bueding, E., and Wilson, W. E., PROC. SOC. EXP. BIOL. AND MED., 1940, **43**, 279.

[6] Artom, C., Fishman, W. H., and Morehead, R. P., PROC. SOC. EXP. BIOL. AND MED., 1945, **60**, 284.

during the second week of serine administration.

There is probably an increase of ammonia after giving each amino acid. The amounts of reducing and bisulfite-binding substances after serine were both markedly elevated in the first days of serine administration, whereas no change was observed after giving *dl*-alanine.

The experimental data of Group *3* (receiving B vitamins plus pyridoxine and *dl*-serine) have been omitted for the sake of brevity. The findings were substantially the same as described for Groups *1a* and *1b*, except that the elimination of bisulfite-binding substances, of ammonia and of serine was more irregular and reached maximum values somewhat later than in Groups *1a* and *1b*.

In some urines, simultaneous determinations of total amino acids and of serine were performed. The differences in the two (which presumably represent amino acids other than serine) were not more than 40 micromoles per rat per day before administering the amino acids. Values found in the urine of rats receiving alanine or serine were often of the same order of magnitude. However, occasionally, and especially in Group *3*, much higher values were found after giving *dl*-serine (83 micromoles on the 1st, 100 micromoles on the pooled urine collected on the 6th, 7th, and 8th days of serine administration).

Discussion. The results of the present investigation corroborate the statement[6] that there are certain characteristic findings in the urine of the animals receiving *dl*-serine. These findings have not been obtained in other animals receiving *dl*-alanine under the same conditions: a point which is interesting in view of the similarity in structure of these 2 amino acids. In this connection, it may also be mentioned that the kidneys of animals receiving *dl*-alanine (studied by Dr. R. P. Morehead) showed no demonstrable pathological lesions.**

Some of the findings, such as the proteinuria, are probably the consequence of the kidney lesions which are already apparent after the first dose of *dl*-serine.[10] The simultaneous presence of sugar might be interpreted similarly. In this respect, one would be tempted to think of a rather specific action of serine on the anatomical structures or on the physiological mechanisms involved in the reabsorption of glucose from the glomerular ultrafiltrate.

In view of the results of the preceding paper, the values for urinary serine obtained in the present study represent probably the elimination of *d*-serine. Consequently, it appears that quite a large fraction (12-25%) of the unnatural isomer has not been metabolized. It has already been pointed out[6] that this fact would be in line with the interpretation of serine injury as being due to a mass action effect of the unmetabolized *d*-serine. However, if this be true, one would expect the clinical recovery to be accompanied by a greater utilization of the administered serine. This expectation is not borne out by the results of the present experiments, in which the excretion of serine persisted at a high level throughout the entire period of the experiment.

Evidence for the formation of some ethanolamine in the body by the decarboxylation of serine has been reported.[11] In the present study, significant amounts of substances reacting as ethanolamine have been found in the urine of the serine-treated animals. This finding occurs rather late (that is, in the period of the experiment when the animals show signs of clinical recovery), and therefore it is not in favor of the possibility that ethanolamine is the agent responsible for the injurious action of serine. In any case, the action of

** *dl*-alanine has been shown to be toxic in pigeons.[7] The action appears to be due to the *d* component.[8] Noxious effects from *d*-alanine have also been observed in rats and guinea pigs. However, in these animals much larger doses are required to produce these effects (700 mg of *d*-alanine per 100 g body weight).[9] Such amounts are 14 times as great as the quantities employed in the present experiments.

[7] Lombroso, U., *Boll. Soc. Ital. Biol. Sp.*, 1933, **8**, 362.

[8] Abderhalden, E., and Tetzner, E., *Z. physiol. Chem.*, 1935, **232**, 79.

[9] Edlbacher, S., and Wiss, O., *Helv. Chim. Acta.*, 1944, **27**, 1060.

[10] Morehead, R. P., Fishman, W. H., and Artom, C., *Am. J. Path.*, 1945, **21**, 803.

[11] Stetten, D., *J. Biol. Chem.*, 1942, **144**, 501.

the intestinal flora does not appear to be essential.[1]

Concerning the increase in bisulfite-binding substances, as suggested previously,[6] it is likely that these substances may include certain products of the metabolism of serine, such as pyruvic acid.[12,13,14] This compound is also formed in the oxidative deamination of alanine. However, no increase in bisulfite-binding substances was found after giving *dl*-alanine.†† This makes less probable the hypothesis of an overproduction of pyruvic acid (*e.g.*, by kidney *d*-amino acid oxidase) as an explanation for the increase in bisulfite-binding substances. Therefore, the hypothesis of a defect in the disposal of pyruvic acid (*e.g.*, as a result of a diminution in kidney cocarboxylase[16]) seems more attractive.

It is hardly necessary to point out that many interpretations other than the ones mentioned are possible; for example, an abnormal concentration of pyruvic acid may arise from an impaired metabolism of carbohydrates and not from serine.

The occasional finding of significant amounts of amino acids other than serine in the urine of animals receiving this amino acid may be of interest. In this connection, one may mention the hypothesis of the formation of glycine in the course of the metabolism of serine,[17,18,19] for which more direct evidence has been presented recently.[20] Another possibility is the production of cystine (or cysteine) from serine.[21,22]

It is difficult to state to what extent the present results explain the mechanism of serine injury. However, as mentioned above, certain findings (protein, sugar, increase in bisulfite-binding and reducing substances) which occur early in the period of serine administration may reasonably be related to the lesion (either as its cause or as its effect), and those findings occurring later (such as the production of ethanolamine) can be thought of as being associated with the recovery of the animal.

Summary. The variations of some chemical constituents in the urine of rats receiving *dl*-serine have been studied quantitatively. The results have been compared with those of similar experiments in which *dl*-alanine has been given. In the serine groups, a significant proportion of the administered amino acid is excreted in the urine. In addition, in the first few days of serine administration, the urine contained protein and sugar, and the amounts of bisulfite-binding and of reducing substances were elevated. The presence of ethanolamine in later periods of the experiment appears probable. None of these changes was observed in the urine of animals receiving *dl*-alanine.

The possible bearing of these results to the injurious action of serine is discussed. At any rate, the present data confirm and extend the indication of chemical changes in the urine which are as typical as the other clinical and pathological alterations found in rats receiving *dl*-serine.

12 Bernheim, F., Bernheim, M. L. C., and Gillespie, A. G., *J. Biol. Chem.*, 1936, **114**, 657.

13 Chargaff, E., and Sprinson, D. B., *J. Biol. Chem.*, 1943, **151**, 273.

14 Binkley, F., *J. Biol. Chem.*, 1943, **150**, 261.

†† Likewise, Waelsch and Miller[15] did not find any increase in urinary keto acids after giving *dl*-alanine.

15 Waelsch, H., and Miller, H. K., *J. Biol. Chem.*, 1942, **145**, 1.

16 Fishman, W. H., and Govier, Wm., *Science*, 1945, **101**, 277.

17 Dakin, H. D., *J. Biol. Chem.*, 1909, **6**, 235.

18 Knoop, F. Z., *Z. physiol. Chem.*, 1914, **89**, 151.

19 Leuthardt, F., and Glasson, B., *Helv. Chim. Acta.*, 1942, **25**, 245.

20 Schemin, D., *Federation Proceedings*, 1945, **4**, 103.

21 Toennies, G., *J. Biol. Chem.*, 1940, **132**, 455.

22 Binkley, F., and Du Vigneaud, V., *J. Biol. Chem.*, 1942, **144**, 507.