ated into protein in rat uterus at the twentieth day when growth is fairly fast, than at the seventh day of pregnancy, when growth has scarcely begun.

SUMMARY

1. The free amino acid content of rat uterus was measured during pregnancy. There was a slight fall after growth began about the ninth day.

2. After injection of α -amino-[^{14}C]isobutyric acid, the ratio $\frac{\text{counts/min./ml. of tissue water}}{\text{counts/min./ml. of serum}}$ did not rise markedly upon the beginning of growth; average values were 6.2 on the sixth, 8.3 on the tenth and 12.5 on the sixteenth day. Marked changes were found in the ratio during the oestrous cycle. After ovariectomy, the ratio fell to 2.6, and rose again to 8.8 after oestradiol injection.

3. Injection of progesterone alone had no effect on the average ratio, and its injection with oestradiol slightly depressed the effect of the latter.

4. In experiments *in vitro* [^{14}C]ornithine was metabolized by rat uterus. Radioactive glutamic acid and proline were formed and incorporated into protein.

Our thanks are due to Dr K. L. Manchester for helpful discussions, and to Mr B. Boon for help with the gas-flow counter. We are indebted, for personal and expenses grants, to the Agricultural Research Council and the Medical Research Council.

REFERENCES

- Akedo, H. & Christensen, H. N. (1962*a*). *J. biol. Chem.* **137**, 113.
 Akedo, H. & Christensen, H. N. (1962*b*). *J. biol. Chem.* **137**, 118.
 Astwood, E. B. (1939). *Amer. J. Physiol.* **126**, 162.
 Awapara, J., Landua, A. J. & Fuerst, R. (1950). *J. biol. Chem.* **183**, 545.
 Bonsnes, R. W. (1947). *J. biol. Chem.* **168**, 345.
 Cawkwell, J. M. (1958). *Biochem. J.* **70**, 248.
 Chibnall, A. C., Mangan, J. L. & Rees, M. W. (1958). *Biochem. J.* **68**, 111.
 Christensen, H. N., Aspen, A. J. & Rice, E. G. (1956). *J. biol. Chem.* **220**, 287.
 Christensen, H. N. & Streicher, J. A. (1948). *J. biol. Chem.* **175**, 95.
 Cole, D. F. (1951). *J. Endocrin.* **7**, 12.
 Davey, C. L. (1957). *Nature, Lond.*, **179**, 209.
 Finch, L. R. & Hird, F. J. R. (1960). *Biochim. biophys. Acta*, **43**, 268.
 Friedberg, F. & Greenberg, D. M. (1947). *J. biol. Chem.* **168**, 411.
 Gale, E. F. & Folkes, J. P. (1953). *Biochem. J.* **55**, 721.
 Halkerston, D. K., Eichhorn, J., Feinstein, M., Scully, E. & Hechter, O. (1960). *Proc. Soc. exp. Biol., N.Y.*, **103**, 796.
 Harkness, M. L. R. & Harkness, R. D. (1954). *J. Physiol.* **123**, 492.
 Kalman, S. M. & Lombrozo, S. E. (1961). *J. Pharmacol.* **131**, 265.
 Krebs, H. A., Eggleston, L. V. & Hems, R. (1949). *Biochem. J.* **44**, 159.
 Levy, A. L. & Chung, D. (1953). *Analyt. Chem.* **25**, 396.
 Manchester, K. L. & Young, F. G. (1960). *Biochem. J.* **75**, 487.
 Meister, A. (1957). *Biochemistry of the Amino Acids*, p. 291. New York and London: Academic Press Inc.
 Michl, H. (1951). *Mh. Chem.* **82**, 489.
 Moore, S. & Stein, W. H. (1954). *J. biol. Chem.* **211**, 907.
 Mueller, G. C. (1953). *J. biol. Chem.* **204**, 77.
 Mueller, G. C., Gorski, J. & Aizawa, Y. (1961). *Proc. nat. Acad. Sci., Wash.*, **47**, 164.
 Noall, M. W. (1960). *Biochim. biophys. Acta*, **40**, 180.
 Noall, M. W., Riggs, T. R., Walker, L. M. & Christensen, H. N. (1957). *Science*, **126**, 1002.
 Robinson, J. R. (1949). *Biochem. J.* **45**, 68.
 Roloff, M., Ratner, S. & Schoenheimer, R. (1940). *J. biol. Chem.* **136**, 561.
 Simkin, J. L. & Work, T. S. (1957). *Biochem. J.* **65**, 307.
 Wood, T. (1957). *Nature, Lond.*, **180**, 39.
 Yarmolinsky, M. B. & de la Haba, G. L. (1959). *Proc. nat. Acad. Sci., Wash.*, **45**, 1721.

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The Aetiology of Canine Cystinuria

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(Received 11 June 1963)

Since the classical case of cystine-stone urolithiasis in an Irish terrier reported by Morris,

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Green, Dinkel & Brand (1935), the cystinuria of canine cystine-stone disease has been attributed to an 'inborn error of metabolism', presumably a block in the conversion of cystine or cysteine into sulphate (Brand, Cahill & Harris, 1935). More

recent semi-quantitative chromatographic investigations (Crane & Turner, 1956; Treacher, 1962) have suggested that the cystinuria is of renal origin and that basic amino acids might also be involved in the anomaly, making the condition similar to human cystinuria in many respects. No fully quantitative investigations have so far been carried out to verify that the cystinuria is of renal origin.

Quantitative studies have been hampered by the lack of suitable methods for the estimation of cystine in body fluids. Simple colorimetric procedures (Looney, 1922; Lugg, 1932; Sullivan & Hess, 1936) have proved insufficiently specific or precise. Microbiological and column-chromatographical techniques are tedious for routine use. The polarographical method of Reed (1942) is relatively simple and reliable but the need for a polarograph might preclude its use in some laboratories.

A new method for the estimation of cystine in urine and plasma has therefore been devised, involving a paper-chromatographic separation followed by spectrophotometry. The method has been used to measure the amounts of cystine in the urine and plasma of dogs with cystine-stone disease and to compare these with those in normal dogs, to provide quantitative data to appraise the theory that cystinuria is of renal origin,

EXPERIMENTAL

Urine was collected from 11 dogs with cystine-stone disease and from 12 clinically normal dogs, as described by Treacher (1963). Blood samples were also taken from seven of each group and treated as described by Treacher (1963).

Determination of creatinine in urines

True creatinine was determined by the standard Jaffé method of Owen, Scandrett, Iggo & Stewart (1954), in which the creatinine is adsorbed on fuller's earth (Lloyd's reagent) to prevent the participation of other Jaffé-positive components of the urine. Recoveries of creatinine added to dog urine were between 96.8 and 100% by this technique.

Determination of cystine

Cystine was determined in native urine, but with plasma it was necessary to deproteinize quantitatively before cystine determinations were made. This was carried out as follows: The apparatus consisted of a glass reservoir sufficient to contain 10 ml. of plasma, connected by an airtight rubber seal to a 5 in. length of $\frac{1}{4}$ in. Visking tubing, sealed at the end. A sample (7 ml.) of plasma was placed in the reservoir and a pressure of 14–15 lb./in.² was applied from a nitrogen cylinder. This pressure was applied gradually to allow the Visking tubing time to soften and expand. The ultrafiltrate was collected in a 250 ml. separating funnel which formed the outer jacket of the apparatus and prevented undue evaporation. The separ-

ing funnel remained at atmospheric pressure because of a small vent in the retaining bung. Five ml. of ultrafiltrate could be obtained over a 6 hr. period. The process completely deproteinized the plasma without changing the cystine concentration.

Unidimensional paper chromatography was used to separate cystine from other amino acids. Cystine was then estimated by reaction with ninhydrin, followed by elution of the spots into glass tubes for spectrophotometry.

Oxidation of cystine to cysteic acid. It was necessary to convert cystine into cysteic acid if a compact clearly-separated spot were to be obtained by paper chromatography. Experiments were carried out to determine the optimum conditions for the oxidation with '100 vol.' (30%) hydrogen peroxide in excess. The reaction went to completion when a small amount of ammonium molybdate was added and the reaction mixture left in the dark for 18–20 hr. The following reagents were used: '100 vol.' (30%) hydrogen peroxide; 0.1% ammonium molybdate solution; water-saturated phenol (125 ml. of water plus 500 g. of phenol); acetone (AnalaR); 0.2% ninhydrin (AnalaR) in acetone; ethanol-water (1:1, v/v); L-cystine (recrystallized).

Procedure: (a) Urine. To 1 ml. of urine were added 0.25 ml. of 30% hydrogen peroxide and 0.1 ml. of 0.1% ammonium molybdate.

(b) Plasma. To 1 ml. of the ultrafiltrate were added 0.1 ml. of 30% hydrogen peroxide and 0.05 ml. of 0.1% ammonium molybdate.

The tubes were stoppered and left overnight in the dark. Samples (25 μ l. or 50 μ l.) were then applied in triplicate to Whatman no. 4 filter paper (18 in. $\times$ 18 in.). The spots were at least $\frac{3}{4}$ in. apart and did not exceed 6 mm. in diameter. The larger sample (50 μ l.) was required with normal dog urines and for plasma, where the cystine concentration was low, whereas 25 μ l. was sufficient for cystinuric urines.

The paper was then developed in phenol-water, by using the descending technique for 16–18 hr., after which it was dried in a cabinet at $30 \pm 2^\circ$ for 7 hr. The paper was then freed from residual phenol by dipping through acetone several times, and dried at 45° for 30 min. It was then dipped through 0.2% ninhydrin in acetone, and the colour was produced by heating for a further hour at $45 \pm 2^\circ$ in a water-saturated atmosphere. The paper was then left in the dark for 16–18 hr. for completion of the colour development.

The cysteic acid spots were cut out together with three paper blanks of the same size, and the paper squares transferred to 5 ml. test tubes. To each was then added 3 ml. of ethanol-water (1:1, v/v), and the tubes were stoppered and inverted intermittently for 30 min., after which elution was complete. The extinctions of the solutions were read at 570 m μ against the extract from the paper blank having the mean extinction reading of the three. Two different standard amounts of cysteic acid were run on each sheet.

Cysteic acid standards. A solution of cysteic acid was freshly prepared: 85 mg. of recrystallized cystine (British Drug Houses, Ltd.) was dissolved in a few millilitres of water to which 3 or 4 drops of conc. hydrochloric acid were added. The solution was then transferred to a 100 ml. volumetric flask, to which 10 ml. of 30% hydrogen peroxide and 5 ml. of 0.1% ammonium molybdate solutions were added before the solution was made up to the mark. The solution was left overnight for the oxidation

to go to completion. A series of dilutions were prepared to give solutions containing about 6–60 mg./100 ml.; 25 μ l. samples of these were spotted in triplicate on Whatman no. 4 paper, and the chromatographic procedure was carried through as described above.

Recovery of cystine. To 1 ml. samples of urines from normal dogs, a series of amounts of cystine were added in 1 ml. of aqueous solution. Samples (25 μ l.) of these mixtures were put through the quantitative procedure, in triplicate. Typical recoveries are given in Table 1.

Table 1. *Recovery of cystine added to dog urine*

Experimental details are given in the text.

Cystine added to 1 ml. of urine (mg.)	Concn. of cystine in mixture (mg./l.)		Recovery of cystine (%)
	Actual	Found	
0	—	1.5	—
0.229	116	112	96.6
0.367	185	180	97.3
0.496	250	239	95.7
0.688	346	338	97.7

Reproducibility. Replicate determinations on the same urine (stored in the deep-freeze) over a period of several weeks gave cystine concentrations agreeing within $\pm 2\%$.

Blank readings lay between 0.002 and 0.015 on the extinction scale.

Sensitivity. The smallest amount of cysteic acid detectable on the chromatograms with this ninhydrin reagent and the Unicam SP. 1400 colorimeter was approx. 0.5 μ g.

RESULTS

The calibration curve of extinction against amount of cysteic acid was linear over the range 0–30 μ g. of cysteic acid ($b = 0.0194$, with s.d. 0.0010; the standard error of the estimate of y from x is 0.0042).

The cystine contents of the samples of dog urine and plasma analysed by the new cystine method are given in Tables 2 and 3. In a study of this nature, 24 hr. urine collections are desirable, to obtain valid comparisons between different animals and between the same animals over periods of time.

Table 2. *Urine and plasma cystine concentrations of dogs with cystine-stone disease*

Experimental details are given in the text. N.T., Not tested.

Subject no.	Breed	Age (years)	Sex	Urine			Plasma concn. of cystine (mg./l.)
				Concn. of cystine (mg./l.)	Concn. of creatinine (g./l.)	Cystine coefficient	
1	Dachshund	2	♂	586	1.62	361	19.6
2	Miniature poodle	4	♂	630	1.05	600	<5.0
4	Labrador	6	♂	237	2.37	100	N.T.
5	Collie cross	6.5	♂	376	2.02	186	<5.0
6	Mongrel	8.5	♂	196	0.53	370	N.T.
11	Dachshund	5	♂	110	0.65	169	N.T.
13	Boxer	6	♂	479	2.49	193	<5.0
17	Dachshund	2	♂	452	1.85	245	N.T.
19	Labrador	6.5	♂	251	2.10	120	6.3
20	Irish terrier	8	♂	123	0.19	648	12.7
22	Corgi	8	♂	125	2.06	61	5.7

Table 3. *Urine and plasma cystine concentrations of clinically normal dogs*

Experimental details are given in the text. N.T., Not tested.

Subject no.	Breed	Age (years)	Sex	Urine			Plasma concn. of cystine (mg./l.)
				Concn. of cystine (mg./l.)	Concn. of creatinine (g./l.)	Cystine coefficient	
3	Dalmatian	0.25	♀	69.2	1.40	49	N.T.
7*	Boxer	7	♀	84.3	2.25	38	N.T.
8	Foxhound	Adult	♂	35.0	1.17	30	10.5
9	Boxer cross	Adult	♂	23.0	0.89	26	N.T.
10	Collie cross	Adult	♂	72.8	1.80	41	N.T.
12	Mongrel	2	♀	16.3	1.45	11	<5.0
15	Mongrel	Adult	♀	76.6	0.93	82	N.T.
16	Mongrel	Adult	♀	57.0	1.00	57	<5.0
18	Boxer	12	♂	111	0.53	210	<5.0
21	Mongrel	8	♂	175	1.22	144	23.0
23	Boxer	3.5	♂	101	1.80	56	16.3
24	Dachshund	5	♂	68.3	1.34	51	12.0

* Case of phosphate urolithiasis.

Such sampling was impossible in this case since all the cystine-stone animals and most of the controls were privately owned, some of them residing long distances away. The next-best alternative was to relate the cystine concentrations in the random samples of urine to the urine creatinine concentration. The 24 hr. creatinine output of the dog is mainly endogenous, and if the animal is on a fixed diet it can be taken as constant. The dog excretes creatinine solely by glomerular filtration. The variation in the 24 hr. creatinine excretion between dogs when related to 24 hr. urine volumes is about $\pm 10\%$. The expression

$$\frac{\text{mg. of cystine/l. of urine}}{\text{g. of creatinine/l. of urine}}$$

has been termed the 'cystine coefficient', and allows a comparison to be made of the levels of cystine excretion, between dogs, based on random urine samples. Cystine coefficients have proved to be fairly reproducible ($\pm 10\%$) on the same dog, with urines taken at different times.

In Tables 2 and 3 both the creatinine concentrations and cystine coefficients have been included, to assist in comparisons between dog urines. In the pathological urines, cystine coefficients range from 11 to 648, with cystine concentrations of 123–630 mg./l. In the normal urines, the coefficients lie between 11 and 210, with cystine concentrations of 11–16 mg./l. Subject 21 was subsequently found to exhibit a chemical cystinuria, although having no history of urolithiasis over the 8 years of its life. It has been included in the normal group.

DISCUSSION

The procedure described above for the quantitative estimation of cystine combines in some measure the high degree of specificity offered by the chromatographic method with the simplicity and speed of the direct colorimetric procedures. The method is much more rapid, where cystine is the sole concern, than ion-exchange methods. Up to 12 samples can be conveniently analysed simultaneously over a 3-night period with only one chromatography tank. The method is less capricious than the direct colorimetric technique and is specific for cystine. The method requires the minimum of apparatus and is easy to set up, provided that the specified chromatographic conditions are rigidly adhered to. The method has been applied to the analysis of urine and plasma and could presumably have wider applications.

Plasma concentrations of cystine in normal dogs (Table 3) were between 16 mg./l. and < 5 mg./l. Values quoted in the literature give a mean value of 9 mg./l., with the 95% range between 5 and 15 mg./l. The plasma cystine concentrations en-

countered in eight cystinuric animals (Table 2), all of which were males, were between 19.6 mg./l. and < 5 mg./l., and these are within normal limits. This adds weight to the two-dimensional chromatographic findings (Treacher, 1962, 1963) in suggesting that the cystinuria of cystine-stone disease in the dog is not in fact due to any abnormal or incomplete metabolism of the amino acid resulting in high plasma concentrations, and thus to an increased excretion. It might be expected that if a metabolic block existed, such as that preventing the oxidation of cystine or cysteine to sulphate (Brand *et al.* 1935), the plasma cystine concentration would be substantially elevated. In human cystinuria it has been suggested that plasma cystine concentrations are in fact slightly below normal (Fowler, Harris & Warren, 1952; Dent, Senior & Walshe, 1954). The results from eight dogs (Table 2) do not suggest that this is so in the dog, but this point would require further investigation.

The quantitative estimation of cystine in cystinuric urines shows cystine concentrations ranging between 123 and 630 mg./l. To make allowance for varying urine concentrations, and the fact that samples of urine were not 24 hr. specimens, the cystine coefficients should be studied. These range from 61 to 648 in the animals with cystine-stone disease (Table 2) and from 11 to 210 in the normal animals (Table 3).

The cystine excretion in dogs appears to follow a similar pattern to that in human populations as described by Harris, Mittwoch, Robson & Warren (1954). In measuring the cystine excretion of 28 patients with cystine-stone disease and 119 of their close relatives, these workers found all levels of excretion ranging from normal values of 25–60 mg./g. up to 700 mg./g. of creatinine. Cases have been reported of daily excretions of over 2 g. of cystine in human cystinuria (Smith, Kolb & Harper, 1959). Though there appears to be a significant difference between the normal dogs and those with cystine-stone disease, it is also obvious that a wide range of variation exists in the degree of cystinuria manifest and there is considerable overlap between normal dogs and those with cystine-stone disease with respect to cystine coefficients. This leads to the possibility that a very high urinary cystine concentration is not the only consideration in the production of cystine stones. Supporting evidence on this point is provided by dog 21, which at 8 years of age had not developed cystine calculi in spite of the presence of a chemical cystinuria. Cystine coefficients have been reproducible to within 20% on the same dog, and yet an animal with a cystine coefficient as high as 210 is to be found in the normal group and one with a value of 61 is in the cystine-stone group. A statistical

comparison of the means of the two groups of cystine coefficients indicated that the probability that the two sets of data belong to different populations is very low ($P < 0.01$), which suggests that a full range of cystine excretion from normal to high can be expected in dog populations. Since canine cystinuria is inherited (Kassell, Brand & Cahill, 1940), an incompletely recessive mode of inheritance would be indicated to explain the existence of normal, intermediate and very high levels of cystine excretion in the same population.

SUMMARY

1. A method for the estimation of cystine as cysteic acid in urine and plasma is described.

2. Cystine concentrations in the urine of 11 dogs with cystine urolithiasis were compared with those of 12 clinically normal dogs. The values have been corrected for differing urine concentrations by dividing by the urinary creatinine in g./l. to give the 'cystine coefficient'.

3. The cystine-urolithiasis group had cystine coefficients between 61 and 600 mg. of cystine/g. of creatinine, whereas the normal animals had coefficients between 11 and 210. Factors other than the level of cystine excretion may be important in stone formation. There was a low statistical probability that the two groups belonged to different populations ($P < 0.01$).

4. Plasma cystine concentrations in seven dogs with cystine-stone disease were not significantly higher than the seven values obtained from normal

animals. This supports the theory that the cystinuria of canine cystine-stone disease is renal in origin.

The author is grateful to the staff of the Liverpool Veterinary Hospital and to the several dog owners and veterinary practitioners who provided specimens and information. Thanks are also due to Professor E. G. White for his guidance and criticism and to the Wellcome Trust for a grant which financed this work. The work forms part of a Ph.D. Thesis submitted to the University of Liverpool.

REFERENCES

- Brand, E., Cahill, G. F. & Harris, M. H. (1935). *J. biol. Chem.* **109**, 69.
 Crane, C. W. & Turner, A. W. (1956). *Nature, Lond.*, **177**, 237.
 Dent, C. E., Senior, B. E. & Walshe, J. M. (1954). *J. clin. Invest.* **33**, 1216.
 Fowler, D. J., Harris, H. & Warren, F. (1952). *Lancet*, **i**, 544.
 Harris, H., Mittwoch, U., Robson, E. & Warren, F. (1954). *Biochem. J.* **57**, xxxiii.
 Kassell, B., Brand, E. & Cahill, G. F. (1940). *Abstr. Pap. Amer. chem. Soc. 100th Meet., Detroit*, p. 29.
 Looney, J. M. (1922). *J. biol. Chem.* **54**, 171.
 Lugg, J. W. H. (1932). *Biochem. J.* **26**, 2144.
 Morris, M. L., Green, D. F., Dinkel, J. H. & Brand, E. (1935). *N. Amer. Vet.* **16**, 16.
 Owen, J. A., Scandrett, F. J., Iggo, B. & Stewart, C. P. (1954). *Biochem. J.* **58**, 426.
 Reed, G. (1942). *J. biol. Chem.* **142**, 61.
 Smith, D., Kolb, F. & Harper, H. (1959). *J. Urol.* **81**, 61.
 Sullivan, M. X. & Hess, W. C. (1936). *J. biol. Chem.* **116**, 221.
 Treacher, R. J. (1962). *Vet. Rec.* **74**, 503.
 Treacher, R. J. (1963). *Res. vet. Sci.* **4**, 556.

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The Participation of Calcium, Adenosine Triphosphate and Adenosine Triphosphatase in the Extrusion of the Granule Proteins from the Polymorphonuclear Leucocyte

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(Received 28 June 1963)

When polymorphonuclear leucocytes are treated with staphylococcal leucocidin in the presence of calcium, proteins known to be present in the cytoplasmic granules are released into the medium, whereas proteins that are found in solution when the cells are homogenized are retained within the cell. The extrusion of the contents of the granules is achieved by a process that involves fusion of the granule with the cell membrane. The omission of

calcium from the medium in which the cells are treated with leucocidin inhibits the extrusion of the granule proteins and simultaneously increases the permeability of the cell to the soluble constituents of the cytoplasm (Woodin & Wieneke, 1963b; Woodin, French & Marchesi, 1963).

The addition of calcium to leucocytes treated with leucocidin in a calcium-free medium induces the extrusion of the granule proteins, but the