reacting haemoglobin (Hb*) is formed. If *p*-chloromercuribenzoate is added almost all the reduced haemoglobin formed is Hb*. At alkaline pH the chief product of photolysis is Hb* even in the absence of *p*-chloromercuribenzoate.

3. At alkaline pH Hb* reverts spontaneously to ordinary haemoglobin with a velocity constant of about 200 sec.$^{-1}$ at 1°.

4. The second-order velocity constant for the combination of Hb* with carbon monoxide is $1{\cdot}8 \times 10^6$ M^{-1} sec.$^{-1}$ at 1° and the activation energy 5·6 kcal.

5. The bearing of these results on the use of flash photolysis in the study of haemoglobin kinetics is discussed.

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REFERENCES

Ainsworth, S. & Gibson, Q. H. (1957). *J. Physiol.* **137**, 26*P*.
Britton, H. T. S. & Welford, G. (1937). *J. chem. Soc.* p. 1848.
Clark, W. M. (1928). *The Determination of Hydrogen Ions*, 3rd ed., p. 200. Baltimore: Williams and Wilkins.
Gibson, Q. H. (1956*a*). *J. Physiol.* **134**, 112.
Gibson, Q. H. (1956*b*). *J. Physiol.* **134**, 123.
Gibson, Q. H. & Roughton, F. J. W. (1955). *Proc. Roy. Soc.* B, **143**, 334.
Gibson, Q. H. & Roughton, F. J. W. (1957). *Proc. Roy. Soc.* B, **146**, 206.
Gutfreund, H. (1949). *Haemoglobin*, p. 197. London: Butterworths Scientific Publications.
Haurowitz, F. (1938). *Hoppe-Seyl. Z.* **238**, 266.
Kolthoff, I. M. (1925). *J. biol. Chem.* **63**, 135.
Riggs, A. F. & Wolbach, R. A. (1956). *J. gen. Physiol.* **39**, 585.
Roughton, F. J. W. (1934). *Proc. Roy. Soc.* B, **115**, 451.
St George, R. C. C. & Pauling, L. (1951). *Science*, **114**, 629.
Sørensen, S. P. L. (1909). *Biochem. Z.* **21**, 131.

The Glutamic Acid and Creatine Content of Cock Seminal Plasma

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There is evidence that the semen of the domestic cock is wholly derived from the reproductive tract proximal to the protruding ejaculatory ducts, so that the possible sources of seminal plasma are the testes, vasa efferentia, epididymides and vasa deferentia (Lake, 1957*a*). The cock lacks the accessory reproductive organs, i.e. prostate and seminal vesicles, characteristic of mammals, and ampullae are absent or ill-defined (Lake, 1957*b*). These organs are the source of a large part of the seminal fluid in mammals. A study of the chemical composition of the seminal plasma of the cock is thus of physiological as well as of comparative interest, and should provide information essential to an understanding of the metabolism of the testis and spermatozoa, especially in the reproductive tract.

In the present investigation the free amino acid content of the seminal plasma has been examined. Gassner & Hopwood (1952) and Larson & Salisbury (1953) have studied this in bull seminal fluid. Since creatine is reported to occur in large amounts in the testes of some mammals (Hunter, 1928; Eggleton, Elsden & Gough, 1943; Ennor & Stocken, 1948) and of certain invertebrates (Greenwald, 1946) it seemed profitable also to examine cock seminal plasma for guanidine bases. Only small amounts of creatine and creatinine have been reported to be present in mammalian semen (Ilyasov, 1933; McKenzie, Miller & Bauguess, 1938).

MATERIAL AND METHODS

Reproductively active Brown Leghorn cocks were maintained in battery cages and received the Poultry Research Centre diet (Bolton, 1958).

Seminal plasma. Samples of uncontaminated semen (0·12–0·6 ml./ejaculate) were collected according to Lake (1957*a*) and centrifuged at 1500 *g* for 15 min. at 2° within 5 min. of collection. The plasma was removed rapidly, before the spermatozoa began to move upwards, and added to 4 vol. of ethanol and heated in boiling water for 15 min. The precipitated protein was removed by centrifuging and 2 vol. of $CHCl_3$ was added to the supernatant to remove lipid. Before chromatography, the aqueous layer was taken to dryness at room temperature in a vacuum desiccator over P_2O_5, and the residue was dissolved in 10% propanol. Appropriate dilutions of the aqueous layer were made for the estimation of non-protein nitrogen, ninhydrin-reacting substances, glutamic acid and creatine. Samples from individual cocks were used for these determinations and for chromatography when possible.

Chromatography. (*a*) Amino acids. One- and two-dimensional chromatography was carried out in phenol saturated with water (0·3% of NH_3) and butanol–acetic acid–water (4:1:5). *iso*Butanol–butan-2-one–water (70:50:30) (Kemble & MacPherson, 1954) was also used as a solvent for unidimensional chromatography. Dinitrophenyl (DNP) derivatives of amino acids were separated by one- and two-dimensional chromatography, with toluene–pyridine–2-chloroethanol–aq. 0·8 N-NH_3 soln. (30:9:18:18) and 2 M-phosphate buffer (pH 6) as solvents (Biserte & Osteux, 1951).

(*b*) Guanidine bases. Unidimensional chromatography, with the solvent systems butanol–acetic acid–water (73:10:17), butanol–formic acid–water (63:20:17) and butanol–aq. NH_3 soln. (sp.gr. 0·88)–water (25:8:17), of Roche, Thoai & Hatt (1954), was used for separating the guanidine bases, which were detected by the three reagents employed by the same workers.

The presence of creatinine was investigated with the Jaffé reaction as applied by Anderson, Williams, Krise & Dowben (1957).

Preparation of dinitrophenyl-amino acids. The method of Levy & Chung (1955) was used.

Determination of non-protein nitrogen content of seminal plasma. Total nitrogen in protein-free extracts was determined by the micro-Kjeldahl method (Chibnall, Rees & Williams, 1943).

Determinations of total ninhydrin-reacting substances and glutamic acid. Initially the total ninhydrin-reacting substances were estimated by the procedure of Moore & Stein (1954); glutamic acid was used as standard. Glutamic acid was estimated after paper chromatography and development of the colour on the paper with ninhydrin (Kay, Harris & Entenman, 1956). Unidimensional, descending chromatograms were run with butanol–acetic acid–water as solvent; running for 48–56 hr. was sufficient to separate glutamic acid from other amino acids. Total ninhydrin-reacting substances were determined similarly by spotting samples on paper and developing the colour without chromatography.

Determination of creatine. The method of Eggleton *et al.* (1943) was used.

RESULTS

A large amount of a substance behaving like glutamic acid was found by paper chromatography of the protein-free extracts of cock seminal plasma with phenol–ammonia and butanol–acetic acid as solvents. Small amounts of alanine, glycine, serine and aspartic acid were also observed but they were not all consistently detected. A substance running in the position of glutathione was also found but it did not respond to the nitroprusside reagent of Toennies & Kolb (1951) and may have been cysteic acid. After prolonged (48–72 hr.) unidimensional chromatography in the above solvents the major component travelled as a discrete spot in the position of glutamic acid and was inseparable from authentic glutamic acid. A similar pattern was obtained with seminal plasma from the upper and lower vas deferens. The absence of glutamine may be especially noted.

Confirmation of the presence of large amounts of glutamic acid was obtained by preparing the DNP derivatives of the amino acids and submitting them to chromatography, when large amounts of a derivative behaving like DNP-glutamic acid was observed; DNP-alanine and DNP-glycine were also detected in traces (Fig. 1), as were three unidentified derivatives. DNP-aspartic acid might not be detected separately from the large amount of DNP-glutamic acid in the sample. Further evidence for the presence of glutamic acid was obtained when some of the 80 % ethanol extracts of seminal plasma were acidified with hydrochloric acid before being dried *in vacuo*. On dissolving the residue and submitting it to chromatography an additional ninhydrin-positive material behaving like the ethyl ester of glutamic acid (Dent, 1948) was observed. Treatment of a glutamic acid solution of approximately the same concentration and under the same conditions produced a substance which was identical chromatographically; in both control and test samples it ran in the position of norvaline (i.e. between valine and leucine–isoleucine) in butanol–acetic acid, but between norvaline and isoleucine in *iso*butanol–butan-2-one.

Visual comparison of samples of seminal plasma with portions of a standard glutamic acid solution after chromatography and development with ninhydrin suggested that the concentration of glutamic acid was of the order of 1 g./100 ml. of seminal plasma. Since the amounts of the other amino acids present were relatively very small the determination of total ninhydrin-reacting substances with glutamic acid as the standard gives a good approximation of the glutamic acid concentration. The results are recorded in Table 1 along with the corresponding figures for non-protein nitrogen, and indicate that the glutamic acid concentration is of the order of 1 g./100 ml. and that amino acids account for about 65 % of the non-protein nitrogen. Confirmation of the former figure was obtained by estimation of the glutamic acid after chromatography in butanol–acetic acid, the chromatograms being run long enough for the

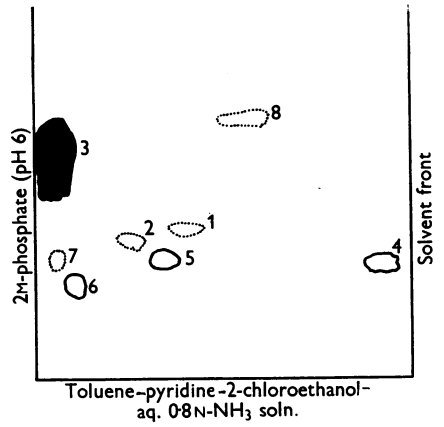

Fig. 1. Two-dimensional chromatogram of DNP derivatives of amino acids. Time of running in 2M-phosphate buffer: 10 hr. (solvent run off paper). 1, DNP-alanine; 2, DNP-glycine; 3, DNP-glutamic acid; 4, dinitroaniline; 5, dinitrophenol; 6, 7, 8, unidentified. Blacked-in spot denotes large concentration; spots shown by dotted lines denote traces and those by solid lines, small amounts.

Table 1. *Non-protein nitrogen and amino acid content of cock seminal plasma*

Ten samples were used.

	Non-protein N (mg./100 ml.)	Amino acids as glutamic acid (mg./100 ml.)	Amino acid N as % of non-protein N
Mean	176·8	1245	67·3
Range	140–275	1040–1980	60·3–79·3
S.D.	37·5	269	6·5

Table 2. *Amino acid and glutamic acid content of cock seminal plasma*

Eight samples were used.

	Amino acids as glutamic acid (mg./100 ml.)	Glutamic acid (mg./100 ml.)	Glutamic acid as % of amino acids
Mean	1207·5	1067·5	88·6
Range	1010–1500	890–1340	84·6–94·2
S.D.	170	136	3·49

Table 3. *Creatine content of cock seminal plasma*

Eight samples were used.

	Non-protein N (mg./100 ml.)	Creatine (mg./100 ml.)	Creatine N as % of non-protein N
Mean	142·8	92·4	21·10
Range	116–208	72–112	17·3–26·7
S.D.	13·9	31·7	2·71

separation of glutamic acid from other amino acids. Table 2 shows that the glutamic acid constitutes about 90% of the total ninhydrin-reacting substances. Differential colour response of individual amino acids may involve a slight error in this estimate.

Examination of the protein-free seminal plasma extracts for guanidine bases with unidimensional chromatography with three different systems revealed the presence of only creatine. The use of the Jaffé reaction failed to reveal any creatinine. The results of the creatine determinations are given in Table 3. About 100 mg./100 ml. of seminal plasma is present, accounting for 20–25% of the non-protein nitrogen. It may be noted that the large amount of glutamic acid present does not interfere with this estimation, and that the creatine content of seminal plasma is approximately 100 times that in the blood plasma of the domestic fowl (Salander & Fisher, 1956).

DISCUSSION

In their study of bull seminal plasma Gassner & Hopwood (1952) found glutamic acid, alanine, glycine, serine and aspartic acid in the approximate amounts 20, 18, 10, 5 and 2 μmoles/100 ml., and Larson & Salisbury (1953) found about ten times these amounts. The same amino acids are found in the seminal plasma of the cock, but the concentration of glutamic acid (about 8 m-moles/100 ml. of plasma) is very much larger and accounts for about 90% of the total amino acids present. The concentration of glutamic acid is about ten times that found in mammalian and avian tissues (Krebs, Eggleston & Hems, 1949), and more than ten times the total amino acid concentration in bull seminal plasma. It must therefore play a large part in the maintenance of the osmotic pressure and of the pH of the seminal plasma.

It was also observed by Gassner & Hopwood (1952) that castration caused the disappearance of amino acids from bull seminal plasma and that the concentration of amino acids other than glutamic acid reach values approaching normal after treatment with testosterone; glutamic acid remained at a very low level. Amino acids also disappeared after vasectomy; again, glutamic acid concentration did not rise on administration of testosterone whereas the other amino acids reached near-normal concentrations. These and subsequent experiments (Hopwood & Gassner, 1957; Gassner & Hopwood, 1957) indicate that the testes are the source of the greater part of the glutamic acid in bull seminal plasma. The very large concentration of glutamic acid in the seminal plasma of ejaculates and of semen obtained directly from the vas deferens of the cock, taken in conjunction with the absence of accessory reproductive organs, suggest the testes are likewise the source of this substance in the domestic cock. The vasa efferentia and epididymides may be involved though they are not very extensive in this species. Since the semen is not diluted with accessory secretions, the glutamic acid concentration remains at a high level in ejaculated semen. Whether the glutamic acid plays a more specific or active part in the life of fowl spermatozoa than that of maintenance of pH and osmotic pressure is not known. Preliminary observations indicate that glutamic acid is not utilized by the spermatozoa *in vitro*, in general agreement with the observations made by Tyler & Rothschild (1951) in the sea urchin, and by Gassner & Hopwood (1952) with bull spermatozoa. The metabolic pathway(s) giving rise to the high glutamic acid concentration is (are) not known.

The large amount of creatine in testis tissue (Hunter, 1928; Eggleton *et al.* 1943; Ennor & Stocken, 1948) would suggest that the high concentration of this substance in cock seminal plasma is mainly of testicular origin. However, some of the creatine may have arisen from the metabolism of the spermatozoa during their stay in the vas deferens. Wajzer & Brochart (1947) have reported the occurrence of both phosphocreatine and phosphoarginine in boar sperm, so that this possibility must be examined.

SUMMARY

1. Free glutamic acid in a concentration of about 1 g./100 ml. has been chromatographically identified in the seminal plasma of the domestic cock. Small amounts of alanine, aspartic acid, glycine, serine and an unidentified ninhydrin-reacting substance have also been detected. About 100 mg. of creatine/100 ml. is also present.

2. About 80% of the seminal plasma non-protein nitrogen is accounted for by glutamic acid plus creatine.

3. It is probable that these substances are mainly of testicular origin and that the glutamic acid plays a major part in maintaining the osmotic pressure and pH.

REFERENCES

Anderson, D. R., Williams, C. M., Krise, G. M. & Dowben, R. M. (1957). *Biochem. J.* **67**, 258.

Biserte, G. & Osteux, R. (1951). *Bull. Soc. Chim. biol., Paris*, **33**, 50.

Bolton, W. (1958). *The Nutrition of Poultry*. Bull. no. 174. London: H.M.S.O.

Chibnall, A. C., Rees, M. W. & Williams, E. F. (1943). *Biochem. J.* **37**, 354.

Dent, C. E. (1948). *Biochem. J.* **43**, 169.

Eggleton, P., Elsden, S. R. & Gough, N. (1943). *Biochem. J.* **37**, 526.

Ennor, A. H. & Stocken, L. A. (1948). *Biochem. J.* **42**, 557.

Gassner, F. X. & Hopwood, M. L. (1952). *Proc. Soc. exp. Biol., N.Y.*, **81**, 37.

Gassner, F. X. & Hopwood, M. L. (1957). *Proc. W. Div. Amer. Dairy Sci. Ass.* 38th Ann. Meet. p. 277.

Greenwald, I. (1946). *J. biol. Chem.* **162**, 239.

Hopwood, M. L. & Gassner, F. X. (1957). *Proc. W. Div. Amer. Dairy Sci. Ass.* 38th Ann. Meet. p. 271.

Hunter, A. (1928). *Creatine and Creatinine*. London: Longmans Green and Co.

Ilyasov, I. (1933). *Trans. vet. Path. Orenburg Vet. Inst.* **1**, 48. See Mann, T. (1954). *The Biochemistry of Semen*. London and New York: Methuen.

Kay, R. E., Harris, D. C. & Entenman, C. (1956). *Arch. Biochem. Biophys.* **63**, 14.

Kemble, A. R. & MacPherson, H. T. (1954). *Biochem. J.* **56**, 548.

Krebs, H. A., Eggleston, L. V. & Hems, R. (1949). *Biochem. J.* **44**, 159.

Lake, P. E. (1957*a*). *J. agric. Sci.* **49**, 120.

Lake, P. E. (1957*b*). *J. Anat., Lond.*, **91**, 116.

Larson, B. L. & Salisbury, G. W. (1953). *J. biol. Chem.* **201**, 601.

Levy, A. L. & Chung, D. (1955). *J. Amer. chem. Soc.* **77**, 2899.

McKenzie, F. F., Miller, J. C. & Bauguess, L. C. (1938). *Res. Bull. Mo. agric. Exp. Sta.* No. 279.

Moore, S. & Stein, W. H. (1954). *J. biol. Chem.* **211**, 907.

Roche, J., Thoai, N. & Hatt, J. L. (1954). *Biochim. biophys. Acta*, **14**, 71.

Salander, R. C. & Fisher, H. (1956). *Proc. Soc. exp. Biol., N.Y.*, **92**, 538.

Toennies, G. & Kolb, J. J. (1951). *Analyt. Chem.* **23**, 823.

Tyler, A. & Rothschild, Lord (1951). *Proc. Soc. exp. Biol., N.Y.*, **76**, 52.

Wajzer, J. & Brochart, M. (1947). *C.R. Acad. Sci., Paris*, **225**, 965.

Calcium and Magnesium Metabolism in Calves

3. ENDOGENOUS FAECAL EXCRETION AND ABSORPTION OF MAGNESIUM*

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It has previously been shown (Smith, 1957, 1958) that the proportion of dietary magnesium excreted in the faeces by milk-fed calves increases as the calves get older. It is not clear whether this increase is due to decreased absorption of magnesium, increased endogenous faecal excretion or in part to both. The values for endogenous faecal excretion reported by Blaxter & Rook (1954) do not give any information on this question since they refer only to young calves.

The present work provides evidence on this matter and on the effect of the level of magnesium in the diet on the efficiency of magnesium absorption.

* Part 2: Smith (1958).

EXPERIMENTAL

General

The calves were all males and all were Friesians except for calf 7A, which was an Angus × Ayrshire crossbred, calf 11A which was an Angus × Shorthorn/Friesian crossbred and calf 18B which was a Hereford × Ayrshire crossbred.

The experiments consisted essentially in the determination of faecal and urinary excretion of magnesium while the calves were receiving low-magnesium synthetic milk and, in some cases, whole milk containing different amounts of magnesium. The calves were reared on whole milk until the experimental periods were started and those kept longer than 9 weeks (2A, 4A, 7A and 11A) were given supplements of iron, copper and manganese from 9 weeks of age and 70 000 i.u. of vitamin D_3/day from 18 to 23 weeks of