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REFERENCES

- Bergmann, M., Fruton, J. S. & Pollok, H. (1939). *J. biol. Chem.* **127**, 643.
- Biggs, R. & Macfarlane, R. G. (1957). *Human Blood Coagulation and its Disorders*, 2nd ed. Oxford: Blackwell's Scientific Publications.
- Clark, W. M. (1928). *Determination of Hydrogen Ions*, 3rd ed., p. 210. London: Ballière, Tindall and Cox.
- Duthie, E. S. & Haughton, G. (1958). *Biochem. J.* **70**, 125.
- Duthie, E. S. & Lorenz, L. L. (1952). *J. gen. Microbiol.* **6**, 95.
- Grob, D. (1949). *J. gen. Physiol.* **33**, 103.
- Kekwick, R. A. & Mackay, M. E. (1954). *Spec. Rep. Ser. med. Res. Coun., Lond.*, no. 286. London: H.M.S.O.
- Lineweaver, H. & Burk, D. (1934). *J. Amer. chem. Soc.* **56**, 658.
- Loeb, L. (1903). *J. med. Res.* **10**, 407.
- Lorand, L. (1954). *Physiol. Rev.* **34**, 742.
- Miale, J. B. (1949). *Blood*, **4**, 1039.
- Milstone, J. H. (1941). *J. Immunol.* **42**, 109.
- Ronwin, E. (1957). *Canad. J. Biochem. Physiol.* **35**, 743.
- Sherry, S. & Troll, W. (1954). *J. biol. Chem.* **208**, 95.
- Smith, W. & Hale, J. H. (1944). *Brit. J. exp. Path.* **25**, 101.
- Smith, W., Hale, J. H. & Smith, M. M. (1947). *Brit. J. exp. Path.* **28**, 57.
- Tager, M. (1948). *Yale J. Biol. Med.* **20**, 369.
- Tager, M. (1956a). *J. exp. Med.* **104**, 675.
- Tager, M. (1956b). *Ann. N.Y. Acad. Sci.* **65**, 109.
- Troll, W., Sherry, S. & Wachman, J. (1954). *J. biol. Chem.* **208**, 85.

Tissue Components of the Domestic Fowl

3. THE NON-PROTEIN NITROGEN OF PLASMA AND ERYTHROCYTES*

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Peters & Van Slyke (1946) define non-protein nitrogen as that nitrogen which is not removed from biological fluids by the usual deproteinizing agents. The same authors (1932) state (obviously with respect to human blood) that 'for non-protein nitrogen tungstic acid and trichloroacetic acid filtrates yield practically identical results'. We have not found satisfactory confirmation of this statement in the literature (cf. Folin, 1922). Greenwald (1915) showed with sheep, ox and pig bloods that both precipitation by 9 vol. of trichloroacetic acid (2.5–22.0%) and direct dialysis gave identical values for non-protein nitrogen. Folin & Wu (1919, cf. p. 86, table 1) compared the non-protein nitrogen of human and fowl bloods after deproteinization by trichloroacetic and tungstic acids; tungstate filtrates contained appreciably less, the difference being especially marked with fowl blood. Hiller & Van Slyke (1922) studied the non-protein nitrogen of slaughter-house bovine blood (previously treated with urease) after deproteinization by a variety of precipitants; the values obtained were by no means identical. Hunter (1949) has indicated the variability of batches of sodium

tungstate in their capacity to precipitate blood ergothioneine (cf. Benedict & Newton, 1929). Puech & Cristol (1929) and Godfried (1939) considered that more amino acid nitrogen is present in blood filtrates when made with trichloroacetic acid than with phosphotungstate. Hunter (1957), in a study of the deproteinization of human blood, plasma, erythrocytes and tissue fluids by heat after appropriate pH adjustment, found that parallel treatments with tungstic acid gave filtrates with lower non-protein nitrogen values in each instance. Christensen & Lynch (1946), comparing trichloroacetic acid and tungstate filtrates of human plasma and erythrocytes, found higher amino acid levels in the former.

Workers on non-protein nitrogen of fowl blood since Folin & Wu (1919) (cf. collated data in Shimer, 1937; Herrmann, 1946a, b; Charkey, Wilgus, Patton & Gassner, 1950; Hsu & Combs, 1952; Hazelwood & Lorenz, 1957) appear, with one exception, to have examined only tungstate filtrates of whole blood; in single instances serum and plasma were analysed. The values found cover a wide range. Despite the observations of Benedict (1915) that non-uric acid chromogens are present in fowl blood, uncertainty exists with regard to

* Part 2: Bell (1957c).

colorimetric measurements of uric acid (cf. Shimer, 1937) by methods which, for mammalian blood, have been adversely criticized, e.g. by Bulmer, Eagles & Hunter (1925) and by Blauch & Koch (1939).

Apart from microbiological assays of seven free amino acids, in young chicks (approx. 500 g. body weight), which have been made by Charkey *et al.* (1950) and by Richardson, Blaylock & Lyman (1953), no information could be found about the nature or distribution of the free amino acids in fowl blood. The data given by Dunn (1949) relating to 'amino acids' in fowl blood are in fact the results of microbiological analyses of acid-hydrolysed whole blood (Dunn, Camien, Malin, Murphy & Reiner, 1949). The findings of Melampy (1948) indexed as 'amino acids in fowl erythrocytes' likewise relate to acidic hydrolysates of whole erythrocytes. Duchateau & Florkin (1954) analysed (microbiologically) an acid hydrolysate of the smaller molecules dialysed from cock plasma. Estimates of total free amino acids of erythrocytes and plasma have been made by Bock (1917) and of plasma by Salander & Fisher (1956).

We have begun an examination of the non-protein nitrogen of fowl blood with two aims: first, as a contribution to comparative biochemistry and, secondly, as part of a clinical investigation into possible changes in the blood chemistry of the nephritic bird. We have adopted routine deproteinization by trichloroacetic acid (9 vols.; 10%, w/v) since this procedure yields the same values as those found in diffusates of heated diluted blood (cf. Bell, 1957*d* for a preliminary account). The sources of the non-protein nitrogen of fowl blood differ from those of mammalian blood especially in the almost negligible contribution afforded in the fowl by urea (Bell, 1957*c*); owing to the relatively large mass of the avian kidney, and its possession of a renal-portal circulation, the bird may have to be *in extremis* before any effect on the small-molecular chemistry of the blood becomes detectable. So far we have been unable to correlate alterations in non-protein nitrogen with known instances of renal dysfunction.

This paper describes the measurement of total non-protein nitrogen, and its partition into 'ninhydrin-nitrogen' and 'residual nitrogen', which is non-protein nitrogen minus 'ninhydrin nitrogen'. All three values have been obtained for plasma and erythrocytes of birds under several different physiological conditions. In addition, we have determined plasma uric acid levels directly by the ultraviolet spectrophotometric uricase method and have qualitatively analysed the free amino acids of both plasma and erythrocytes of cocks and hens by high-voltage paper electrophoresis.

EXPERIMENTAL AND RESULTS

Brown Leghorn fowls, presumed healthy, were allowed free access to water and food (Bell, 1957*a*). Starved birds were deprived of food, but not water, for 36 hr. Blood samples were taken from the wing vein, at about mid-day; for chemical measurements the blood was citrated (about 2 mg./ml.) and for haematocrit determinations it was oxalated (Wintrobe, 1942). Hens were diagnosed as non-laying by observing the absence from the plasma of the 'lipophosphoprotein complex' (McIndoe, 1957) and by their failure to lay within 10 days after blood sampling.

Non-protein nitrogen. Citrated whole blood or plasma (1 vol.) was added to 10% (w/v) trichloroacetic acid solution (9 vol.) and the mixture was shaken and filtered after keeping it at room temperature for 15 min. Filtrate samples were analysed by the method of Chibnall, Rees & Williams (1943), with their $\text{CuSO}_4\text{-Na}_2\text{SeO}_4\text{-K}_2\text{SO}_4$ catalyst (as tablets, 0.25 g., from Thompson and Capper Ltd., Liverpool 10).

Comparison of non-protein nitrogen measurements on trichloroacetic acid filtrates with those on diffusates from dialysed boiled blood (pH 6.4). Equal volumes of the same blood samples were treated (a) by trichloroacetic acid precipitation and analysis as above, and (b) by Hunter's (1956) method followed by dialysis as follows. A suitable piece of cellophan tubing was weighed, washed in water, and pressed between filter paper to remove loose water and weighed again; the difference, indicating the amount of water absorbed by the cellophan, was required for subsequent accurate dilution of the blood. After tying one end of the sac, a suitable volume of 0.01 N-acetic acid was introduced, followed by one-fourth of the volume of blood. The sac was then tied at the open end, and immediately inverted to mix the contents and placed in a glass-stoppered tube containing a volume of water sufficient to give a blood 'dilution' after dialysis of 1 in 20, taking into account the water absorbed by the cellophan. The stoppered glass vessel and its contents were then heated in a boiling-water bath until the blood proteins were fully coagulated (3-4 min.). After removal from the bath the tube was rotated on rollers at an angle of about 30° to aid mixing of the contents. Samples of the diffusate were taken for analysis after 16 hr., experiments having shown that equilibration was then complete. These diffusates gave no precipitate with trichloroacetic acid. The analytical results are given in Table 1.

Comparison of non-protein nitrogen in whole-blood filtrates obtained by four methods of deproteinization. The methods

Table 1. *Comparison of non-protein nitrogen in trichloroacetic acid filtrates and diffusates of hen blood*

Blood specimen	For details see text. Non-protein N (mg./100 ml. of blood)		
	Heat-deproteinization at pH 6.4		Trichloroacetic acid filtrate
	Undialysed	Diffusate	
1	61, 63	42, 43	42, 41
2	63	45, 47, 46, 46	48, 47
3	84	55, 54, 54, 55	55, 56
4	—	39, 38	38, 38

used were (a) 1 in 10 dilution with 10% trichloroacetic acid, as above; (b) 1 in 10 dilution and boiling at pH 6.4 (Hunter, 1956); (c) 1 in 20 tungstic acid precipitation (Van Slyke & Hawkins, 1928); (d) 1 in 10 metaphosphoric acid precipitation (Folin & Denis, 1916). From the results (Table 2), and from those of the dialysis experiment, it was decided to adopt trichloroacetic acid as the most satisfactory protein precipitant.

Erythrocyte volume. This was deduced from the fully packed cell volume (3200 g at 5°), with Bell's (1957b) correction of 6% for the proportion of plasma occluded in the column of erythrocytes. Clarkson & Maizels (1955) and Gourley (1957) have, by different methods, assessed the occluded fowl plasma at 3-4% and 7-8% respectively. Typical results are given in Table 3. The white cells (vol.

about 1.0-1.5%) of the spun blood were neglected in the chemical analyses.

Non-protein nitrogen analyses of whole blood, plasma and erythrocytes. The analyses were done on whole blood and on plasma. The values for the erythrocytes were obtained with help of the erythrocyte volume determined as described above. The results, from birds of different sexes and in physiological states, are given in Table 4.

Uric acid and urea. Plasma was analysed for uric acid by the method of Dubbs, Davies & Adams (1956). Both plasma urea and whole-blood urea gave indistinguishable results by the method of Bell (1957c). Sex, age, the nutritional state and egg production all affect the uric acid level. Urea, on the other hand, is not affected by factors which influence the plasma uric acid.

Table 2. Comparison of whole-blood non-protein nitrogen values found after four methods of deproteinization

For details see text. Each horizontal row of figures represents analyses by the four different methods on an individual bird.

			Non-protein N (mg./100 ml. of blood) found after deproteinization by method stated				
Age of birds (years)	Sex	State	9%	Heat	Tungstic†	Metaphosphoric‡	
			trichloroacetic acid	(pH 6.4)*	acid	acid	
2	M	Starved 48 hr.	47	58	32	48	
			60	78	36	64	
			65	77	40	61	
			60	72	47	63	
			60	51	52	64	
			Mean	58.4	67.2	41.4	60.0
2	M	Fed	66	89	50	63	
			64	73	51	65	
			80	97	49	82	
			64	62	30	60	
			58	88	54	62	
			Mean	66.4	81.8	46.8	66.4
1	F	Fed	47	55	—	—	
			44	56	—	—	
			50	71	—	—	
			—	72	48	—	
			—	66	53	—	
			—	62	47	—	
			Mean	—	63.6	—	—
* Hunter (1956).			† Van Slyke & Hawkins (1928).		‡ Folin & Denis (1916).		

Table 3. Erythrocyte volumes as percentages of whole blood in birds from the same inbred line

Sex of birds	Condition of birds	No. of observations	Erythrocyte volume (%)		
			Mean	Range	S.D.
F	Laying, fed	29	25.13	19.0-30.9	2.52
F	Laying, starved	6	30.58	29.5-34.2	2.34
F	Non-laying, fed	12	30.58	28.5-33.3	1.50
F	Non-laying, starved	6	30.52	26.6-33.2	2.80
M, F	Immature* (10 weeks), fed	10	24.4	22.8-25.7	0.84
M	Fed	6	34.7	29.9-39.0	4.43
M	Starved	6	38.0	33.3-40.9	3.10

* No significant difference in values is found between sexes at this age (10 weeks).

Significant differences between groups of hens:

Fed vs. starved
Laying. D.F. = 33
 $t = 4.85, P < 0.01$

Laying vs. non-laying
Fed. D.F. = 39
 $t = 7.01, P < 0.01$

'Ninhydrin nitrogen' and 'residual nitrogen'. With slight modifications, the method of Yemm & Cocking (1955) was satisfactorily applied to trichloroacetic acid filtrates from plasma and blood. If it was desired to keep the solution for analysis for some time, the pH was first brought to approx. 5 by dropwise addition of 3M-NaOH followed by aq. 5% acetic acid; phenolphthalein and methyl red were used as internal indicators. Otherwise the acid filtrate was used directly. The filtrate (1 ml.), neutralized or not as required, was diluted to a final volume of 5 ml. and of this solution 1 ml. samples were used for analysis. The reagents and conditions were as specified by Yemm & Cocking, except that fresh potassium cyanide (A.R.) solution was prepared daily and 5M-acetate buffer (Chibnall, Mangan & Rees, 1958) was used. The reagents were mixed immediately before use. Since proline and hydroxyproline were present as only minor components of the fluids, absorptions were measured only at 570 m μ .

Standards run simultaneously contained 1.4, 2.8 and 4.2 μ g. of N/ml. and were prepared from recrystallized

glycine dissolved in the appropriate concentration of trichloroacetic acid. Variations in the concentration of the latter did not affect the absorption at 570 m μ .

Corpuscular concentrations of ninhydrin N (NN) were calculated in the same way as the non-protein N concentrations. Residual N (RN) concentrations were found by subtracting NN from non-protein N. The concentrations in plasma and erythrocytes are given in Table 6.

High-voltage paper electrophoresis of amino acids. De-proteinized filtrates (Hunter, 1956), 1 in 10, were prepared from plasma and packed erythrocytes (3 200 g, temp. 5°) obtained from the bloods of hens and cocks. In order to detect amino acids present in small amount the filtrates had to be concentrated to one-tenth of their bulk.

A few modifications of the electrophoretic-separation technique (Gross, 1955, 1956) enhanced the degree of resolution and reliability of identification of the separated acids. The choice of four suitable background electrolyte solutions (namely: pH 2.0, 0.75M-formic acid; pH 4.5, 0.2M-sodium acetate adjusted with 0.2M-NaOH; pH 5.2,

Table 4. *Non-protein nitrogen values for whole blood, plasma and erythrocytes of hens (17-24 months) from the same inbred line, showing the effect of starvation for 36 hr. on layers and non-layers*

Values for immatures (10 weeks) and fed and starved cocks are included for comparison.

Sex and no. of observations	Condition of birds	Non-protein nitrogen (mg./100 ml. of tissue)								
		Whole blood			Concn. of plasma N			Concn. of erythrocyte N		
		Mean	Range	S.D.	Mean	Range	S.D.	Mean	Range	S.D.
F, 29	Laying, fed	40.7	33-53	4.53	17.2	12-21	2.21	109	73-147	16.4
F, 6	Laying, starved	46.5	40-56	5.56	17.3	16-23	2.90	113	99-132	11.0
F, 12	Non-laying, fed	50.1	39-56	4.79	20.0	16-27	4.33	118	87-135	13.0
F, 6	Non-laying, starved	45.3	42-51	3.30	19.3	18-22	2.06	105	93-117	7.4
M, 5 + F, 5*	Immature (10 weeks) fed	56.2	50-67	5.00	22.7	14-27	2.78	160	143-188	11.8
M, 6	Fed	57.0	51-61	4.12	26.7	24-29	1.19	114	105-125	7.1
M, 6	Starved	49.3	37-58	7.5	22.0	20-25	1.50	94	59-114	17.6

* No significant difference in values was found between sexes in immature fowls.

Significant difference between groups of hens:

	Fed vs. starved	Fed vs. starved	Laying vs. non-laying	Immature vs. non-laying
	Laying. D.F. = 33	Non-laying. D.F. = 16	Fed. D.F. = 39	Fed. D.F. = 32
Whole blood non-protein N (%)	$t = 2.73, P < 0.01$	$t = 2.17, P < 0.05 > 0.02$	$t = 5.86, P < 0.01$	$t = 6.09, P < 0.01$
Plasma non-protein N	—	—	$t = 2.50, P < 0.02$	$t = 2.75, P < 0.01$

Table 5. *Plasma uric acid and urea levels of hens and cocks in various states*

Sex and state of birds	Hens, laying		Hens, non-laying		Hens, immature	Cocks	
	Fed	Starved	Fed	Starved	Fed	Fed	Starved
Uric acid (mg./100 ml. of plasma)							
Mean and no. of samples	2.27 (15)	1.41 (9)	5.40 (9)	1.50 (3)	4.38 (9)	2.86 (14)	2.26 (5)
Range	0.8-5.3	0.3-2.2	2.9-9.9	0.8-2.1	2.2-6.3	1.1-5.3	1.4-2.7
S.D.	1.28	0.76	2.44	0.42	1.33	0.93	0.55
Calc. means for 100 ml. of whole blood	1.7	1.1	3.8	1.0	3.3	1.9	1.4
Urea (mg./100 ml. of blood or plasma*)							
Mean and no. of samples	2.23 (7)	2.50 (8)	2.80 (10)	2.83 (6)	2.38†	2.01 (14)	—
Range	1.86-2.6	1.8-3.7	1.9-3.3	2.2-3.7	1.4-3.4†	1.3-2.8	—
S.D.	0.39	0.21	0.58	0.20	0.76†	0.5	—

* These values for blood and for plasma were the same.

† Values from Bell (1957a).

Table 6. *Total non-protein nitrogen, 'ninhydrin nitrogen' and 'residual nitrogen' concentrations (mg./100 ml.) in plasma and erythrocytes from groups of 12 laying, non-laying and immature (6 weeks) hens*

State of birds	...	...	...	...	...	...	...	Laying	Non-laying	Immature
Plasma										
Non-protein N							Mean	15.4	18.9	19.4
							Range	12-21	12-23	17-25
							s.d.	2.9	2.1	2.3
Ninhydrin N							Mean	7.8	8.3	9.1
							Range	5.4-11.6	4.6-11.5	4.0-14.3
							s.d.	1.6	1.8	2.9
Residual N							Mean	7.5	11.5	8.2
							Range	2.8-13.4	8.7-15.7	4.0-15.0
							s.d.	3.0	2.2	3.8
Ratio residual N/ninhydrin N (means)								0.96	1.59	0.90
Erythrocytes										
Non-protein N							Mean	113	105	144
							Range	95-139	72-132	133-159
							s.d.	14.3	14.6	8.3
Ninhydrin N							Mean	31.9	43.0	50.5
							Range	23.0-40.5	29.8-61.0	40.0-64.0
							s.d.	5.3	9.3	7.5
Residual N							Mean	79	62	93
							Range	66-104	31-80	80-116
							s.d.	12.3	15.2	11.4
Ratio residual N/ninhydrin N (means)								2.48	1.44	1.84

Significant differences between layers and non-layers

Plasma: Non-protein N; D.F. = 22, $t = 2.611$, $P < 0.02 > 0.01$ Residual N; D.F. = 22, $t = 3.161$, $P < 0.01 > 0.001$ Erythrocytes: Ninhydrin N; D.F. = 22, $t = 2.51$, $P < 0.05 > 0.02$ Residual N; D.F. = 22, $t = 2.704$, $P < 0.02 > 0.01$ Table 7. *Qualitative analysis by high-voltage electrophoresis of the distribution of amino acids between erythrocytes and plasma*

The number of plus signs indicate the estimated relative concentrations. A minus sign indicates that the substance in question could not be detected; (+ ?) indicates a tentative identification.

Compound	Hen		Cock	
	Cells	Plasma	Cells	Plasma
Alanine	+	+	++	++
4-Aminobutyrate	-	-	-	+
Arginine	+++	+	++	++
Aspartate	++	+	++	+
Cystine-cysteine	(+ ?)	-	++	+
Glutamate	++	++	++	++
Glutamine	-	-	(+ ?)	-
Glycine	+++	+	+++	++
Histidine	+	-	+	Trace
Hydroxyproline	-	-	+	Trace
Leucine-isoleucine	++	+	{ ++	+
Lysine	++	Trace	{ +	+
Methionine	+	+	++	+
Ornithine	-	-	(+ + ?)	(+ + ?)
Proline	+	+	++	++
Serine	++	++	+++	++
Taurine	++++	Trace	++++	Trace
Threonine	++	+	Trace	++
Tryptophan	+	+	-	-
Tyrosine	-	-	++	-
Valine	++	++	+	+

0.05M-citric acid adjusted with aq. 0.05M- NH_3 soln.; pH 9.2, 0.05M-sodium tetraborate) widened the range of specific separations. For example, at pH 4.5 4-amino-butyrate, glutamate and aspartate are best separated, whereas for lysine, arginine and histidine, pH 5.2 is most efficient. The positions at pH 2.0 of ornithine, lysine, glycine, alanine, leucine-isoleucine (inseparable), tryptophan and taurine are characteristic for these acids, and so are the positions of cysteic acid, cystine-cysteine (inseparable), taurine, proline, asparagine and serine at pH 9.2. To help in sorting out the neutral amino acids, two-dimensional electrograms were run first at pH 2.0, and dried, then moistened by spraying carefully with borate solution of pH 9.2, turned through 90° and finally run at pH 9.2 for a suitable period of time. The complete experiment, involving 20 amino acids, can be done in 80 min., when applying a potential gradient of 100v/cm. in either direction. By this technique the following acids were unambiguously separated and identified: tyrosine, phenylalanine, methionine, threonine, serine, leucine-isoleucine and valine. It also showed unmistakably the presence of histidine, and confirmed the results obtained by unidimensional runs with respect to the other acids. The application of the two-dimensional technique was made possible by the construction of equipment capable of taking filter-paper sheets 20 in. $\times$ 12 in., instead of strips 20 in. $\times$ 4 in., which in the past had proved unsatisfactory. Unidimensional electrophoresis was carried out under the following conditions: at pH 2.0, 150v/cm., 8 mA/cm., 12° (cooling water), 25 min.; at pH 4.5, 100v/cm., 10 mA/cm., 12°, 20 min.; at pH 5.2, 80v/cm., 12 mA/cm., 14°, 25 min.; at pH 9.2, 100v/cm., 10 mA/cm., 12°, 40 min. Whatman paper no. 3 MM was used throughout, with an applied pressure of 1.5 lb./in.². Where necessary the electrophoretic results were checked by paper chromatography, with specific reagents in addition to ninhydrin (0.5%, w/v) in acetone [acidified with 3% (v/v) acetic acid for alkaline electrograms]. Specific reagents used were: the modified Sakaguchi reagent (Acher & Crocker, 1952) for arginine; Pauly's reagent (Dent, 1948) for histidine and tyrosine; Ehrlich's reagent (Smith, 1953) for tryptophan; the platinum tetraiodide reagent (Toennies & Kolb, 1951) for methionine; and the isatin reagent (Smith, 1953) for proline and hydroxyproline. The solvent used for the separation of leucine-isoleucine by paper chromatography was *tert*.-butanol-butan-2-one-water, (4:4:2, by vol.; Boissonas, 1950).

The identity of taurine, which was found to be present in a surprisingly high proportion, was checked by the electrophoretic position at various pH values, by a specific reagent (Toennies & Kolb, 1951), and by the R_F values in various solvent systems, including acetone-water (9:1, v/v; Lindberg, 1955). The results are given in Table 7.

DISCUSSION

Erythrocyte volumes. As previously observed (cf. Lorenz, 1954; Bell, 1957*b*), the erythrocyte volume of fed laying hens was significantly lower than that of fed non-layers. In birds, alterations in erythrocyte volume are believed to be related to the state of the oestrogen-androgen balance (cf. Blacher, 1925; Herrick, Lockhart, Martin &

Nusser, 1954), but whether the changes in the ratio of cells to plasma are a result of alterations in plasma volume has not been satisfactorily established (cf. Lorenz, 1954; Ramsey & Campbell, 1954). The rise in erythrocyte volume which we found after a starvation period of 36 hr. in the laying hen (Table 3), and which was followed by a 40% decrease in subsequent egg production in the ensuing 14 days, may be due to a diminished hypothyseal production of gonadotrophic hormone resulting in follicular atresia and a consequently lessened release of oestrogen(s) (Hosoda, Kaneko, Mogi & Abe, 1955). Among the hens examined, starvation of non-layers did not affect the erythrocyte volume (Table 3), although in cocks it caused a marked rise. The significance to whole blood non-protein nitrogen measurements of alterations in erythrocyte volume is clear, for the erythrocyte non-protein nitrogen concentration is five to six times that in the plasma (Table 4). The rise in blood non-protein nitrogen observed by Shimer (1937) when laying hens were starved can now be explained by increase in the proportion of circulating erythrocytes rich in non-protein nitrogen and not by a rise throughout the whole tissue (Table 3). Salander & Fisher (1956) and Bell (1957*b*) have already pointed out the necessity for recognizing that small-molecular blood components, in the fowl, need not necessarily be equally distributed between the rapidly fluctuating formed and unformed elements and that the results of whole-blood analyses may easily be misinterpreted.

Non-protein nitrogen of whole blood and of plasma. As did Folin & Wu (1919), we find that trichloroacetic acid filtrates contain more non-protein nitrogen than do tungstate filtrates (Table 2). Hunter's (1956) method does not remove all the material which is non-diffusible and precipitable by trichloroacetic acid (Table 1), contrary to his (1957) finding with human blood. The nature of the substances which co-precipitate with protein-tungstate is not known; it is likely that they are bases.

Plasma uric acid and urea. Since the main end-product of avian nitrogen metabolism is uric acid, the plasma levels might be expected to reflect differences in diet and in nutritional state, as observed by Bollman & Schlotthauer (1936) in turkeys and by Oppenheimer & Kunkel (1943) in fowls, and also differences in the extent and direction of protein metabolism in the bird. The higher plasma uric acid values of non-layers as compared with layers presumably indicate an increased catabolism of surplus 'nitrogen' resulting from depressed synthesis of protein, especially as such differences are not evident in starved birds. The wide range of values may be due to differences in egg production or depend on whether the non-layers were or were not moulting.

Unpublished work by W. M. McIndoe has confirmed Benedict (1915) who showed that the erythrocytes contain no detectable uric acid; measurements on whole blood must therefore take the erythrocyte volume into account. If our plasma figures are translated into the corresponding concentrations for whole blood (Table 5) these prove to be appreciably lower than many reported from colorimetric observations, e.g. the collated mean values quoted by Shimer (1937), which range between 1.35 and 6.39 mg./100 ml. of blood. On the other hand, our values for immature birds agree with those found by Hsu & Combs (1952) for plasma by Brown's (1945) method, but are lower than those of Bolton (1947), who used the Christman & Ravitch (1932) method. The plasma values of Levine, Wolfson & Lenel (1947) for 'young chickens' are somewhat higher; Wolfson, Levine & Tinsley (1947) and Wolfson, Levine & Huddlestun (1947) estimated the plasma non-uric acid chromogen as about 16% of the apparent total (similar to that in man and dogs) in agreement with Benedict's (1915) postulate of about 20%.

Urea was found to be equally distributed between cells and plasma, in agreement with Jacobs & Grassmann (1937) (cf. Hunter, 1946), who noted that equality of urea distribution is not found in all races of birds examined. Apart from the observation of Bemporad & Iorio (1945) that fowl muscle can split arginine, the kidney (Clementi, 1945) is the sole organ in the fowl known to produce urea from arginine. [This work of Clementi was unknown to one of us at the time of writing an earlier paper (Bell, 1957c), where it was stated that the site of the formation of avian blood urea was unknown.] It seems reasonable to regard avian blood urea as being maintained through a fairly constant reabsorption from the urine and as a non-participant in the general nitrogenous metabolism of the animal. Whether muscle produces appreciable amounts of urea *in vivo* is not known.

Concentration of erythrocyte non-protein nitrogen (Table 4). In the laying hen the concentration of erythrocyte non-protein nitrogen shows a large scatter, and this led us to see if it were possible to correlate the concentration with, among other things, egg production. From Fig. 1 it will be seen that such a correlation does exist and is statistically significant. The more eggs the birds laid in an arbitrarily chosen period of 6 days after blood sampling the lower was the concentration; it seemed likely, therefore, that the erythrocytes can lose nitrogenous substances to meet an extra-vascular demand.

Although the concentrations of erythrocyte non-protein nitrogen of fed and starved hens, both laying and out of lay, were not significantly different, it is noteworthy that immature (10 weeks)

birds showed concentrations very much higher than fed non-layers. The possibility that, in these rapidly growing birds, the erythrocytes were carrying extra materials yielding non-protein nitrogen cannot be ruled out.

For comparison parallel measurements on cocks are recorded; it is not proposed to comment on these now.

Free amino acids of plasma and erythrocytes (Table 7). The intracellular occurrence and apparent concentration of certain free amino acids is of interest. Microbiological-assay methods (Charkey *et al.* 1950; Richardson *et al.* 1953) have indicated the presence of arginine, lysine, methionine, tryptophan, histidine, threonine and valine in blood and plasma; in preliminary experiments we have added alanine, aspartate, cystine-cysteine, glutamate, glycine, leucine and isoleucine, proline, serine and especially taurine, to the list for hens and cocks. In addition, tryptophan was detected in both cells and plasma of the hen but not of the cock, tyrosine in cells of the cock and 4-aminobutyrate in cock plasma. With the exception of methionine and valine, those amino acids known to be nutritionally essential to the fowl, as well as aspartate, appear to be more concentrated in the cells than in the plasma. The absence of glutamine is noteworthy as it is present in relatively large amounts in mammalian blood; this may be due to the high requirement of this amide for uric acid synthesis. Although free taurine is known to be widely distributed, we are not aware that it has previously been found in avian blood. In the fowl embryo its presence, especially in the heart, has been shown by Gonzales & Awapara (1955) and its formation from [35 S]sulphate (Machlin, Pearson & Denton, 1954; Gonzales & Awapara, 1955; Lowe & Roberts, 1955) and from [35 S]-cysteine and

Fig. 1. Relationship between erythrocyte non-protein N and the number of eggs laid by a group of six hens during a period of 6 days. Blood was sampled on the fourth day. Regression is -4.388 ; $P < 0.01 > 0.001$.

-methionine (Machlin, Struglia & Pearson, 1955) has been demonstrated. Machlin & Pearson (1957) have studied the effect of injection of [35 S]taurine into chicks and have demonstrated its incorporation into several excretory conjugates, one of which is partly derived from ornithine. This last substance may be formed by a process analogous to ornithuric acid synthesis, involving renal argininolysis (cf. Crowdle & Sherwin, 1923; Hunter & Dauphinée, 1925). How taurine is formed and retained within the fowl erythrocyte is unknown.

The detection, so far in cock plasma alone, of 4-aminobutyrate is of interest in view of the attention at present being paid to its activity in the cortical structure of the mammalian brain (Bazemore, Elliot & Florey, 1956; Iwama & Jasper, 1957; McLennan, 1957; Curtis & Phillis, 1958), where it has been found in several species and is formed from L-glutamate by a decarboxylase (Wingo & Awapara, 1950; Tallan, Moore & Stein, 1954; Roberts & Frankel, 1951). Whether it is an accidental component of nutritional origin (in the cock) cannot yet be decided. It may be noted that homopantothein found in human urine contains 4-aminobutyric acid in place of the 3-aminopropionic acid of pantothein (Biserte, Plaquet & Boulanger, 1955). 4-Aminobutyrate has not until now been reported in birds.

'Ninhydrin nitrogen' and 'residual nitrogen' of plasma of fed hens (Table 7). In plasma the non-protein nitrogen is approximately equally divided between NN and RN although in layers compared with non-layers RN is significantly lower. This can partly be accounted for by differences in uric acid levels of nitrogen (Table 5). The NN figures correspond well with the amino acid values found by Bock (1917) and Salander & Fisher (1956), who used respectively the Van Slyke and the Danielson method. Only a fraction of the RN (mg./100 ml. of plasma) is accountable for from published average values, as follows: urea nitrogen, 1.5 mg. (Bell, 1957c, Table 5); uric acid nitrogen, 0.1–3.3 mg. (Table 5); creatine nitrogen, 0.3 mg. (Salander & Fisher, 1956); a total of about 5.1 mg. of RN as a maximum. Therefore 2.4–6.4 mg. of RN is of unknown origin, but might be accounted for by basic amino acids and substances giving less than '100 % of colour' with ninhydrin.

'Ninhydrin nitrogen' and 'residual nitrogen' of erythrocytes of fed hens (Table 7). Until more is known about the chemistry of the NN and RN fractions of the erythrocyte non-protein nitrogen little can be discussed about the results of our measurements beyond noting that the RN of the immature birds was very high. As with plasma, the RN is only partially accounted for by observations known to the authors. These are as follows, the

figures indicating RN in mg./100 ml. of erythrocytes: urea nitrogen, 1.5 (Table 4); creatine nitrogen, 0.3 (Salander & Fisher, 1956); adenosine triphosphate nitrogen, 9 (Rapoport & Guest, 1941); ergothioneine nitrogen, 4 (Hunter, 1928); glutathione nitrogen, 7 (Chanton, 1932), 20 (Grunert, Meyer & Phillips, 1951). The discrepancy in erythrocyte RN cannot wholly be accounted for by basic amino acids.

We conclude that the erythrocytes of the fowl may contain a portion of the animal's nitrogen pool and play a part in the carriage and supply of nitrogenous small molecules to extravascular metabolic centres.

SUMMARY

1. Non-protein nitrogen was measured on blood or plasma deproteinized by trichloroacetic acid. Diffusates of blood which had been diluted and boiled at pH 6.4 gave the same values. Deproteinization by tungstate removed some diffusible non-protein nitrogen. 'Ninhydrin nitrogen' was also measured on trichloroacetic acid filtrates.

2. Erythrocyte non-protein nitrogen is five to six times that of plasma. The erythrocyte portion of the blood volume fluctuates with the physiological state and varies in relation to sex and nutrition; it therefore biases measurements of non-protein nitrogen made on whole blood.

3. Significantly higher levels of whole blood non-protein nitrogen were found in the first of each of the following pairs: (a) starved and fed laying hens; (b) fed and starved non-laying hens; (c) fed non-laying and fed laying hens; (d) immature and non-laying hens. These differences are almost entirely due to differences in erythrocyte volume.

4. Erythrocyte non-protein nitrogen concentrations were markedly higher in immature birds than in adults.

5. Plasma non-protein nitrogen concentrations were significantly higher (a) in non-laying than in laying fed hens, (b) in immature than in non-laying hens.

6. In a single experiment involving six hens a statistically significant correlation was found between their erythrocyte nitrogen concentrations and the number of eggs laid during the 6 days subsequent to blood sampling.

7. Uric acid plasma levels were related to sex and to the physiological and nutritional states.

8. In filtrates of erythrocytes and plasma of hens and cocks, qualitatively analysed by high-voltage paper electrophoresis, 18 free 2-amino acids were detected with evidence of higher concentrations of some in the erythrocytes, namely arginine, aspartate, cystine-cysteine, glycine, histidine,

leucine-isoleucine and lysine. A high concentration of taurine in the cells and the apparent absence from the blood of glutamine are noteworthy. 4-Aminobutyrate was found in cock plasma.

9. The non-protein nitrogen of plasma and of erythrocytes was fractionated into 'ninhydrin nitrogen' and 'residual nitrogen'. In immature birds the amounts of both the erythrocyte 'ninhydrin' and 'residual nitrogen' were at levels distinctly higher in mature animals. The erythrocyte 'ninhydrin nitrogen' of non-layers was higher than that of layers, whereas the reverse held for the erythrocyte 'residual nitrogen'. The plasma 'residual nitrogen' and the plasma non-protein nitrogen of non-layers was significantly higher than that of layers. The plasma 'ninhydrin nitrogen' corresponds with values found by others for 'amino acids.'

10. We suggest that the fowl erythrocytes form part of the nitrogen pool of the animal and can contribute 'non-protein nitrogen' to meet extra-vascular needs.

REFERENCES

- Acher, R. & Crocker, C. (1952). *Biochim. biophys. Acta*, **9**, 704.
- Bazemore, A., Elliot, K. A. C. & Florey, E. (1956). *Nature, Lond.*, **178**, 1052.
- Bell, D. J. (1957a). *Biochem. J.* **66**, 137.
- Bell, D. J. (1957b). *Quart. J. exp. Physiol.* **42**, 410.
- Bell, D. J. (1957c). *Biochem. J.* **67**, 33.
- Bell, D. J. (1957d). *Biochem. J.* **67**, 20p.
- Bemporad, G. & Iorio, C. (1945). *Boll. Soc. ital. Biol. sper.* **20**, 537.
- Benedict, S. R. (1915). *J. biol. Chem.* **20**, 633.
- Benedict, S. R. & Newton, E. B. (1929). *J. biol. Chem.* **82**, 5.
- Biserte, G., Plaquet, R. & Boulanger, P. (1955). *Bull. Soc. Chim. biol., Paris*, **37**, 831.
- Blacher, L. J. (1925). *Biol. gen.* **2**, 436.
- Blanch, M. B. & Koch, F. C. (1939). *J. biol. Chem.* **130**, 443.
- Bock, J. C. (1917). *J. biol. Chem.* **29**, 191.
- Boissonas, R. A. (1950). *Helv. chim. acta*, **33**, 1966.
- Bollman, J. L. & Schlotthauer, C. F. (1936). *Amer. J. dig. Dis.* **3**, 483.
- Bolton, W. (1947). *J. agric. Sci.* **37**, 316.
- Brown, H. (1945). *J. biol. Chem.* **158**, 601.
- Bulmer, F. M. R., Eagles, B. A. & Hunter, G. (1925). *J. biol. Chem.* **53**, 17.
- Chanton, L. R. (1932). *C.R. Soc. Biol., Paris*, **110**, 556.
- Charkey, L. W., Wilgus, H. S., Patton, A. R. & Gassner, F. X. (1950). *Proc. Soc. exp. Biol., N.Y.*, **73**, 21.
- Chibnall, A. C., Mangan, J. L. & Rees, M. W. (1958). *Biochem. J.* **68**, 111.
- Chibnall, A. C., Rees, M. W. & Williams, E. F. (1943). *Biochem. J.* **37**, 354.
- Christensen, H. N. & Lynch, E. L. (1946). *J. biol. Chem.* **163**, 741.
- Christman, A. A. & Ravwitch, S. (1932). *J. biol. Chem.* **95**, 115.
- Clarkson, E. M. & Maizels, M. (1955). *J. Physiol.* **129**, 476.
- Clementi, A. (1945). *Boll. Soc. ital. Biol. sper.* **20**, 461, 464, 466, 468.
- Crowdle, J. H. & Sherwin, C. P. (1923). *J. biol. Chem.* **55**, 363.
- Curtis, D. R. & Phillis, J. W. (1958). *Nature, Lond.*, **182**, 323.
- Dent, C. E. (1948). *Biochem. J.* **43**, 168.
- Dubbs, C. A., Davies, F. W. & Adams, W. S. (1956). *J. biol. Chem.* **218**, 497.
- Duchateau, G. & Florkin, M. (1954). *Arch. int. Physiol.* **42**, 205.
- Dunn, M. S. (1949). *Physiol. Rev.* **29**, 219.
- Dunn, M. S., Camien, M. N., Malin, R. B., Murphy, E. A. & Reiner, P. J. (1949). *Univ. Calif. Publ. Physiol.* **8**, 293.
- Folin, O. (1922). *Physiol. Rev.* **2**, 460.
- Folin, O. & Denis, W. (1916). *J. biol. Chem.* **27**, 491.
- Folin, O. & Wu, H. (1919). *J. biol. Chem.* **38**, 81.
- Godfried, E. G. (1939). *Biochem. J.* **33**, 955.
- Gonzales, F. & Awapara, J. (1955). *Exp. Cell Res.* **9**, 355.
- Gourley, D. H. R. (1957). *Amer. J. Physiol.* **190**, 536.
- Greenwald, I. (1915). *J. biol. Chem.* **21**, 61.
- Gross, D. (1955). *Nature, Lond.*, **176**, 72.
- Gross, D. (1956). *Nature, Lond.*, **178**, 29.
- Grunert, R. R., Meyer, J. H. & Phillips, P. H. (1951). *Amer. J. Physiol.* **165**, 568.
- Hazelwood, R. L. & Lorenz, F. W. (1957). *Endocrinology*, **61**, 520.
- Herrick, E. H., Lockhart, C. H., Martin, E. P. & Nusser, W. L. (1954). *Trans. Kans. Acad. Sci.* **57**, 483.
- Herrmann, G. R. (1946a). *Proc. Soc. exp. Biol., N.Y.*, **61**, 229.
- Herrmann, G. R. (1946b). *Proc. Soc. exp. Biol., N.Y.*, **61**, 302.
- Hiller, A. & Van Slyke, D. D. (1922). *J. biol. Chem.* **53**, 253.
- Hosoda, T., Kaneko, T., Mogi, K. & Abe, T. (1955). *Proc. Soc. exp. Biol., N.Y.*, **88**, 502.
- Hsu, P. T. & Combs, G. F. (1952). *Arch. Biochem. Biophys.* **38**, 9.
- Hunter, A. & Dauphinée, J. A. (1925). *Proc. Roy. Soc. B*, **97**, 227.
- Hunter, F. R. (1946). *Proc. Soc. exp. Biol., N.Y.*, **61**, 204.
- Hunter, G. (1928). *Biochem. J.* **22**, 4.
- Hunter, G. (1949). *Canad. J. Res. E*, **27**, 230.
- Hunter, G. (1956). *Biochem. J.* **62**, 28p.
- Hunter, G. (1957). *J. clin. Path.* **10**, 161.
- Iwama, K. & Jasper, H. H. (1957). *J. Physiol.* **138**, 365.
- Jacobs, M. H. & Grassmann, H. N. (1937). *Biol. Bull., Wood's Hole*, **73**, 387.
- Levine, R., Wolfson, W. Q. & Lenel, R. (1947). *Amer. J. Physiol.* **151**, 186.
- Lindberg, B. (1955). *Acta chem. scand.* **9**, 1323.
- Lorenz, F. W. (1954). *Vitam. & Horm.* **12**, 235.
- Lowe, J. P. & Roberts, E. (1955). *J. biol. Chem.* **212**, 477.
- Machlin, L. J. & Pearson, P. B. (1957). *Arch. Biochem. Biophys.* **70**, 35.
- Machlin, L. J., Pearson, P. B. & Denton, C. A. (1954). *J. biol. Chem.* **205**, 859.
- Machlin, L. J., Struglia, L. & Pearson, P. B. (1955). *Arch. Biochem. Biophys.* **59**, 326.

- McIndoe, W. M. (1957). *Biochem. J.* **67**, 19P.
 McLennan, H. (1957). *J. Physiol.* **139**, 729.
 Melampy, R. (1948). *J. biol. Chem.* **175**, 589.
 Oppenheimer, E. H. & Kunkel, H. G. (1943). *Johns Hopk. Hosp. Bull.* **73**, 40.
 Peters, J. P. & Van Slyke, D. D. (1932). *Quantitative Clinical Chemistry*, vol. 2, p. 537. London: Baillière, Tyndall and Cox.
 Peters, J. P. & Van Slyke, D. D. (1946). *Quantitative Clinical Chemistry*, vol. 1, pp. 632 *et seq.* London: Baillière, Tyndall and Cox.
 Puech, A. & Cristol, P. (1929). *Ann. Méd.* **25**, 43.
 Ramsey, W. N. M. & Campbell, E. A. (1954). *Biochem. J.* **58**, 313.
 Rapoport, S. & Guest, G. M. (1941). *J. biol. Chem.* **138**, 269.
 Richardson, L. R., Blaylock, L. G. & Lyman, C. M. (1953). *J. Nutr.* **49**, 21.
 Roberts, E. & Frankel, S. (1951). *J. biol. Chem.* **188**, 789.
 Salander, R. C. & Fisher, H. (1956). *Proc. Soc. exp. Biol., N.Y.*, **92**, 538.
 Shimer, S. R. (1937). *Tech. Bull. N.H. agric. Exp. Sta.*, No. 69.
 Smith, I. (1953). *Nature, Lond.*, **171**, 43.
 Tallan, H. H., Moore, S. & Stein, W. H. (1954). *J. biol. Chem.* **211**, 927.
 Toennies, G. & Kolb, J. J. (1951). *Analyt. Chem.* **23**, 823.
 Van Slyke, D. D. & Hawkins, J. A. (1928). *J. biol. Chem.* **79**, 739.
 Wingo, W. J. & Awapara, J. (1950). *J. biol. Chem.* **187**, 267.
 Wintrobe, M. M. (1942). *Clinical Haematology*. London: Kimpton.
 Wolfson, W. Q., Levine, R. & Huddleston, B. (1947). *J. clin. Invest.* **26**, 995.
 Wolfson, W. Q., Levine, R. & Tinsley, M. (1947). *J. clin. Invest.* **26**, 991.
 Yemm, E. W. & Cocking, E. C. (1955). *Analyst*, **80**, 209.

The Fermentation of Lactate and Acrylate by the Rumen Micro-organism LC

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Washed cell suspensions of the anaerobic coccus LC isolated from the rumen of sheep by Elsdén, Volcani, Gilchrist & Lewis (1956) ferment lactate (Ladd, 1957, 1959) and acrylate (Lewis & Elsdén, 1955) to hydrogen, carbon dioxide, acetate, propionate, butyrate and valerate. Lewis & Elsdén (1955) showed that both washed cell suspensions and acetone-dried powders of LC reduce acrylate to propionate in an atmosphere of hydrogen, and they suggested that acrylate or a derivative of acrylate is an intermediate in the conversion of lactate into propionate.

In this paper we describe the fermentation of lactate and acrylate by cell-free extracts of LC and demonstrate a requirement of each fermentation for 'sparking' by a number of high-energy compounds. Evidence is also presented for a coupled oxidation-reduction reaction between lactate and acrylate. The effects of various inhibitors on the fermentations are reported. The results give added support to the direct pathway of propionate formation in this organism.

Most of this work has been communicated to a meeting of the Biochemical Society (Ladd & Walker, 1958; Walker & Ladd, 1958).

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MATERIALS AND METHODS

Organism. Cultures of LC were grown in 10 l. glass aspirators containing 8 l. of medium by the method of Walker (1958*a*). The cells were harvested on the Sharples Super-centrifuge and washed once with deoxygenated water.

Preparation of cell-free extracts. A paste of washed cells was crushed without abrasive in the Hughes bacterial press previously cooled to approx. -20° (Hughes, 1951). The frozen material so obtained was suspended in 50 ml. of water and held at 37° for 5 min. with occasional shaking to ensure complete thawing. Cell debris was removed by centrifuging at 14 000 *g* in the International refrigerated centrifuge for 20 min. at 0° . The clear, green supernatant, which contained the activity, was dispensed into test tubes and stored under air at -15° .

Manometric methods. All experiments were carried out at 37° under an atmosphere of pure N_2 or H_2 . Single side-bulb manometer cups were used for most experiments. The enzyme preparation was placed in the side bulb and, after temperature equilibration, was added to the substrate mixed with 200 μ moles of buffer and water to a final volume of 2.6 ml. in the main compartment. When rates of H_2 output or uptake were required, 0.1 ml. of 10*N*-NaOH and a piece of folded filter paper were placed in the centre well and used for the absorption of CO_2 . When CO_2 estimations were necessary, double side-bulb cups were used, the second side bulb containing 0.2 ml. of CO_2 -free 2*N*-NaOH and a piece of folded filter paper. After gas exchange had