Onderstepoort J. Vet. Res. (1964), 31 (1), 77–90
Printed in the Republic of S. Afr. by the Government Printer, Pretoria

CONTRIBUTIONS TO THE STUDY OF BLOOD CONSTITUENTS IN DOMESTIC ANIMALS IN SOUTH AFRICA. I: NORMAL VALUES FOR METHAEMOGLOBIN REDUCTASE, ERYTHROCYTE FRAGILITY, BLOOD GLUTATHIONE, PLASMA CATALASE AND THE TOTAL ERYTHROCYTE PYRIDINE NUCLEOTIDES IN SHEEP

ADRIANA M. WAGNER, Veterinary Research Institute, Onderstepoort

INTRODUCTION

Recent studies on the pathogenesis of geeldikkop (*Tribulosis ovis*) and enzootic icterus in sheep in South Africa have indicated the existence of certain fundamental biochemical lesions in affected animals. These disturbances have been correlated with a low-grade chronic and subclinical selenium intoxication (Brown, 1962, 1963; Brown & De Wet, 1962). Both syndromes are marked by intravascular haemolysis, increased erythrocyte fragility and disturbances of the methaemoglobin reductase systems in the red cells. Elevated plasma catalase values have been mentioned by Brown (1963) as occurring in the geeldikkop syndrome. An empirical evaluation of the integrity of the erythrocyte metabolic systems, known as the " fragility index ", has been used for establishing the existence of the red cell lesions in latent or subclinical cases of either syndrome (Brown, 1962, 1963).

In order to proceed with research into the mechanisms of intravascular haemolysis in these conditions and with investigations into some of the other interesting features of these diseases encountered by Brown and his co-workers, it was necessary to establish " normal values " for some of the tests used on sheep and normal blood levels of the various enzymes and co-factors under consideration. It was of importance also to establish whether such values as are available in the literature for some of these estimations are applicable to sheep under South African farming conditions. Furthermore it was essential to know whether significant differences existed between apparently healthy and clinically normal sheep raised in areas where these diseases do not occur and those from areas in which the two syndromes are enzootic. Brown (1959, 1962, 1963), Brown & De Wet (1962) and Brown, le Roux & Tustin (1960) have indicated that a large percentage of apparently healthy sheep from the Karoo areas do in fact exhibit profound biochemical disturbances manifested in extreme cases by severe atrophic hepatic cirrhosis, subacute or chronic hepatitis, moderate to severe anaemia and bone marrow hypoplasia.

In this and subsequent papers, details of the " normal values " for some of the tests used in sheep and normal blood levels of the factors of interest for this work will be presented.

Received for publication on 16 September, 1963.—Editor

Materials and Methods

The sheep used in these studies fall into two broad groups.

(*a*) *Group* 1: Fully grown Merino sheep drawn from the pool of animals available for research work at this Institute. Approximately 75 per cent of these animals had been purchased within the last two years from farmers in the areas where geeldikkop and enzootic icterus are enzootic, i.e. the Karoo, notably the Laingsburg and Beaufort West districts. The remainder were obtained over the same period from farms on the Transvaal Highveld. All these animals have at some time during the period mentioned been immunized and used for potency testing of vaccines against bluetongue, heartwater, blackquarter, anthrax and enterotoxaemia. The sheep are maintained at the Institute on a ration of lucerne hay with concentrate supplementation containing " corn and cob " meal, lucerne hay meal, blood meal, bone meal, salt and urea.

(*b*) *Group* 2: Fully grown Merinos and Dorpers bred and raised at the experimental farm of the University of Pretoria. This farm is in a grass pasture area in which geeldikkop and enzootic icterus have never been observed. Besides the natural grazing the animals received supplements in the form of soya hay and silage. These sheep had within the last two years received routine inoculations against bluetongue and enterotoxaemia.

Mild, subclinical verminosis was common to both groups of animals.

The following determinations were done by the methods indicated in parentheses immediately following the procedure mentioned: methaemoglobin reduction test (Brewer, Tarlov & Alving, 1960), erythrocyte fragility (Brown, 1963), glutathione, using whole blood (Grunert & Phillips, 1951) and plasma catalase (Dobkin & Glantz, 1958). An Evans Electro-Selenium portable model "A" photo-electric colorimeter was used throughout, with the appropriate Ilford light filters. The total pyridine nucleotides present in erythrocytes was determined using the fluorimetric method of Levitas, Robinson, Rosen, Huff & Perlzweig (1947) with an Evans Electro-Selenium fluorimeter.

Oxalated blood was used for glutathione, red cell pyridine nucleotides and erythrocyte fragility determinations, heparinized blood for catalase estimation and blood collected in A.C.D. solution as described by Brewer *et al.* (1960) for the methaemoglobin reduction test. In all instances determinations were commenced within half an hour after sample collection.

Methaemoglobin reduction test values are expressed as percentage of methaemoglobin remaining unreduced at the end of the test period (Brewer *et al.*, 1960). Erythrocyte fragility values given are those found for erythrocytes in 0·7 per cent saline (Brown, 1963). Glutathione is expressed as mg of this compound per 100 ml of whole blood, as in the original procedure used, total pyridine nucleotide values being presented in the same manner (Levitas *et al.*, 1947).

Considerable difficulty has been experienced in the direct application of the Dobkin & Glantz (1958) method for plasma catalase to ovine blood owing to the very high levels of this enzyme normally present. In the procedure of these authors a unit of catalase activity is defined as the amount of enzyme catalyzing the decomposition of 1×10^{-3} m.eq/ml of H_2O_2, at pH 6·8 and with an incubation period of 10 minutes at 25° C, using an initial substrate concentration of 10×10^{-3} m.eq/ml of H_2O_2 ($=0{\cdot}01$ N H_2O_2). The equation given by these workers for the photometric estimation of this enzyme is $U/S \times 10 =$ catalase units. Since the method depends on the determination of H_2O_2 remaining undecomposed at the end of the test period, values

for U in this equation (photometer readings for the test samples, when S represents the standard readings) will be very small in the face of large amounts of catalase in the test samples. Units of catalase for sheep blood will thus be less than those given for humans, if this calculation is used. In actual fact there is considerably more of this enzyme present in ovine than in human blood.

In order to overcome this difficulty a calibration curve for catalase was constructed over the range of 0 to 10 units, using the definition of a unit as given by the original authors, and thus also their original photometric equation. The readings from the curve constructed represent in actual fact values obtained from an equation of the form: $10-(U/S\times 10)$=catalase units.

Using this method of calcuation the arbitrary units of Dobkin & Glantz (1958) are converted into figures which are simpler to interpret, and make comparisons between species easier.

In the statistical evaluation of the results obtained, normal values were considered as 80 per cent of the population evenly distributed about the median as suggested by King & Wootton (1959). Any percentage of the population falling within certain limits can be determined from a cumulative relative frequency curve as suggested by J. J. C. Sion (1953, unpublished method and observations) for such data, when the population is sufficiently homogeneous.

Results

The cumulative relative frequency curves and histograms constructed from the results obtained from the various determinations performed are presented for each group of sheep at the end of this paper. The conclusions drawn are shown in Table 1.

Discussion

As seen from the histograms (Fig. 1–5) the distribution curves were in almost all instances of the " skew " type. The method of expressing the results as a standard deviation about a mean cannot, therefore, be employed.

Table 1 does not contain the upper and lower 1 per cent limits as shown by King & Wootton (1959) in their discussion on this topic since in the words of these authors " In clinical practice any single result falling *outside* the 10 per cent limits is considered suspicious; a result which is *outside* the 1 per cent limit is almost certainly abnormal." The 1 per cent limits are readily determined by inspection of the cumulative relative frequency curves.

The populations of Groups 1 and 2 were selected to be as homogeneous as possible with respect to age, management, nutritional state and degree of parasitic infestation. Neither group is entirely homogeneous with regard to sex, since although the majority of sheep used were wethers, a small percentage of ewes was included. Although at least 75 per cent of the animals in Group 1 were originally purchased from farms in areas where the syndromes mentioned earlier are enzootic, there is some element of doubt as to the origin of the remainder of this group. These animals were supplied to this Institute by various Agricultural Field Stations in the Transvaal. Although the relevant records are not available it is believed that many of these sheep were also in actual fact bred on Karoo farms. For this reason they have been included in Group 1. Group 2 is entirely homogeneous in this respect, the sheep having been bred and raised in the Pretoria district.

TABLE 1.—*Ranges found for the blood constituents, etc., determined*

Determination	Figures shown by Median (50%)		Ranges per group					
			80%		10% Lower		10% Upper	
	Group 1	Group 2	Group 1	Group 2	Group 1	Group 2	Group 1	Group 2
Methaemoglobin reductase...	46·0 (n=147)	38·5 (n=78)	22–61·5	19·5–49·5	5·0–21·9	5·0–19·4	61·6–75	49·6–75·0
Erythrocyte fragility.....	21·5 (n=105)	7·5 (n=82)	7·5–52·0	2·5–31·0	2·5–7·4	0–2·5	52·1–72·5	31·1–52·5
Glutathione (whole blood)....	14·5 (n=100)	20·0 (n=61)	7·5–22·5	12·75–26·75	2·5–7·4	2·5–12·7	22·6–32·5	26·8–32·5
Plasma catalase.....	0·8 (n=152)	8·9 (n=51)	0·2–1·65	8·63–9·1	0·125–0·19	8·375–8·62	1·66–2·875	9·2–9·375
Total erythrocyte pyridine nucleotides	3·05 (n=108)	3·0 (n=51)	2·37–3·72	2·4–3·6	2·25–2·36	2·25–2·3	3·73–5·25	3·7–4·25

The animals of both groups were selected as " normal " on grounds of appearing healthy and free from obvious signs of disease on clinical examinations.

From the results presented here it is clear that for all the determinations performed, with the exception of total pyridine nucleotides in the erythrocytes, differences are observed in the " normal values " obtained from the two groups of sheep. This difference is particularly striking in the case of the plasma catalase values, where the normal range for sheep bred and raised in the Transvaal is approximately eight times higher than that for sheep emanating largely from the Karoo.

The methaemoglobin reductase systems of the erythrocytes of animals in the latter group appear to be considerably less efficient than those of red cells from animals raised in the Transvaal. Furthermore there appears to be a significant difference in erythrocyte fragility between the two groups, the greatest fragility in 0·7 per cent saline being exhibited by red cells from the animals originating from the Karoo.

Some small difference between the whole blood glutathione levels of the two groups of animals is apparent, the animals from the Karoo in general showing somewhat lower values than the Transvaal sheep.

Blood glutathione levels for sheep are given by one team of Australian workers (Kidwell, Bohman, Wade & Hunter, 1959) as $32 \cdot 2 \pm 4 \cdot 2$ mg per 100 ml of blood. No significant differences attributable to either age or sex were found by these authors. The values reported in this paper for glutathione in both groups of experimental animals are noticeably lower than those found by Kidwell and co-workers.

The results of this work serve to emphasize a number of important points. In the first instance they indicate the differences which may be present between different geographical population groups of a single species. The fallacy of accepting so-called " normal values " for a species regardless of environmental influences is, therefore, apparent.

At this Institute many sporadic, fatal cases of enzootic icterus have occurred amongst sheep maintained for experimental work over the last three decades. In many instances the records of these animals are no longer available but in the more recent cases their origin is traceable to the Karoo areas in which this syndrome or geeldikkop are enzootic. In the past, the occurrence of these sporadic cases at Onderstepoort was one of the most perplexing aspects of enzootic icterus, especially since some sheep succumbed to the disease at periods of up to five years after leaving the Karoo. Brown and his co-workers have postulated a chronic low-grade selenium intoxication possibly coupled with other as yet unknown factors as the basic disease entity of which geeldikkop and enzootic icterus form two distinct clinical manifestations. They have observed that these syndromes may be precipitated by severe and non-specific stress conditions (Brown, 1962, 1963). The findings reported here not only serve to confirm the earlier observations of these workers that many fundamental biochemical differences exist between sheep considered as normal healthy animals in the Karoo and those considered as normal elsewhere in this country, but also illustrate that many of the biochemical lesions might indeed remain for years after animals have been removed from the affected areas—even in the absence of obvious internal pathological lesions. Herein may well be found the explanation for the sporadic cases of enzootic icterus encountered amongst sheep at this Institute.

SUMMARY

Blood analysis figures obtained from two groups of sheep of different geographical origin are presented. The one group emanated from the Karoo and the other from the Transvaal grassland. Substantial differences between groups for all factors excepting pyridine nucleotides are demonstrated. It is suggested that these differences may reflect basic disturbances in apparently healthy Karoo sheep connected with the aetiology of geeldikkop and enzootic icterus.

REFERENCES

BREWER, C. J., TARLOV, A. R. & ALVING, A. S., 1960. Methaemoglobin Reduction Test. A new simple *in vitro* test for identifying primaquine-sensitivity. *Bull. W. H. O.* No. 22, 633

BROWN, J. M. M., 1959. Advances in Geeldikkop (*Tribulosis ovis*) Research. 3. The epizootology of Geeldikkop. *J. S. Afr. Vet. Med. Ass.* 30, 403.

BROWN, J. M. M., 1962. Advances in Geeldikkop (*Tribulosis ovis*) Research. 5. Aetiological factors in Geeldikkop and Enzootic Icterus. *J. S. Afr. Vet. Med. Ass.* 33, 493.

BROWN, J. M. M., 1963. Biochemical lesions in the pathogenesis of Geeldikkop (*Tribulosis ovis*) and Enzootic Icterus in sheep in South Africa. *Ann. N.Y. Acad. Sci.*, Vol. 104, Art. 2, 504.

BROWN, J. M. M., LE ROUX, J. M. W. & TUSTIN, R. C., 1960. Advances in Geeldikkop (*Tribulosis ovis*) Research. 4. The pathology of Geeldikkop—Part 1. *J. S. Afr. Vet. Med. Ass.* 31, 179.

BROWN, J. M. M. & DE WET, P. J., 1962. A preliminary report on the occurrence of selenosis in South Africa and its possible role in the aetiology of Tribulosis (*Geeldikkop*), Enzootic Icterus and some other disease conditions encountered in the Karoo areas. *Onderstepoort J. Vet. Res.* 29, 111.

DOBKIN, G. B. & GLANTZ, M. D., 1958. Colorimetric determination of plasma catalase. *Clin. Chem.* 4, 316.

GRUNERT, R. R. & PHILLIPS, P. H., 1951. A modification of the nitro prusside method of analysis for glutathione. *Arch. Bioch.* 30, 217.

KING, E. J. & WOOTTON, I. D. P., 1959. Micro-analysis in Medical Biochemistry. 3rd ed. London: J. and A. Churchill, Ltd.

KIDWELL, J. F., BOHMAN, V. R., WADE, M. A. & HUNTER, J. E., 1959. Investigation of blood glutathione levels in sheep. *Growth* 22, 63.

LEVITAS, N., ROBINSON, J., ROSEN, F., HUFF, J. W. & PERLZWEIG, W. A., 1947. A rapid fluorimetric method for the determination of the total pyridine nucleotides in the red blood cells. *J. Biol. Chem.* 167, 169 and 151.

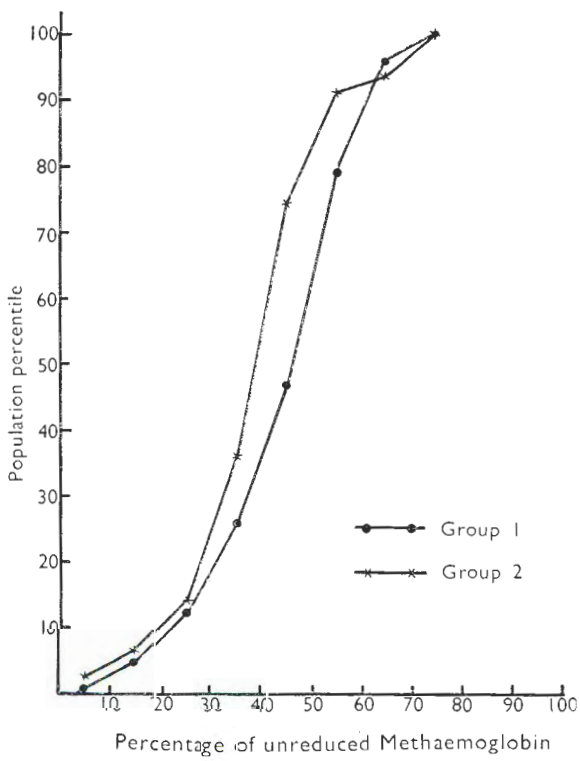

FIG. 1.—Methaemoglobin reduction test

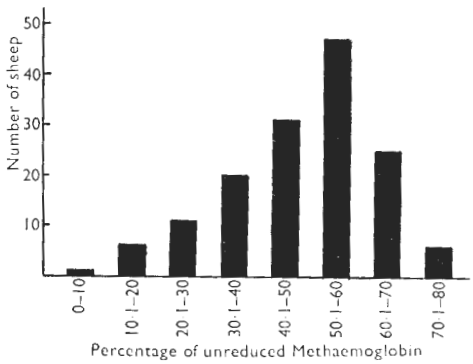

FIG. 1a.—Methaemoglobin reduction test—Group 1.

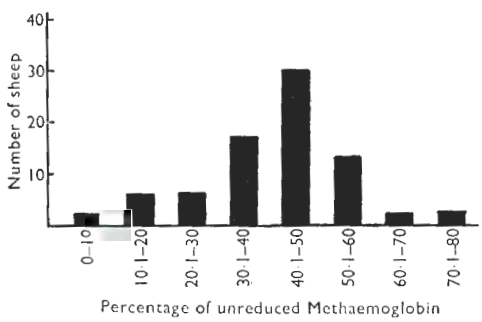

FIG. 1b.—Methaemoglobin reduction test—Group 2.

4940617—6

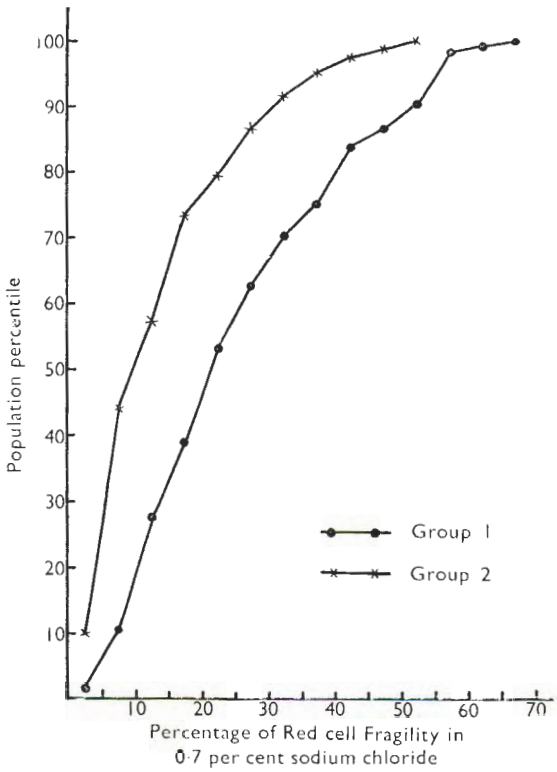

FIG. 2.—Red cell fragility test.

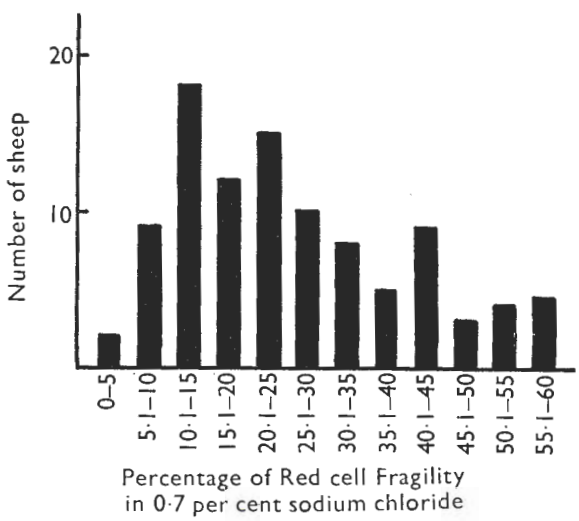

FIG. 2a.—Red cell fragility test—Group 1.

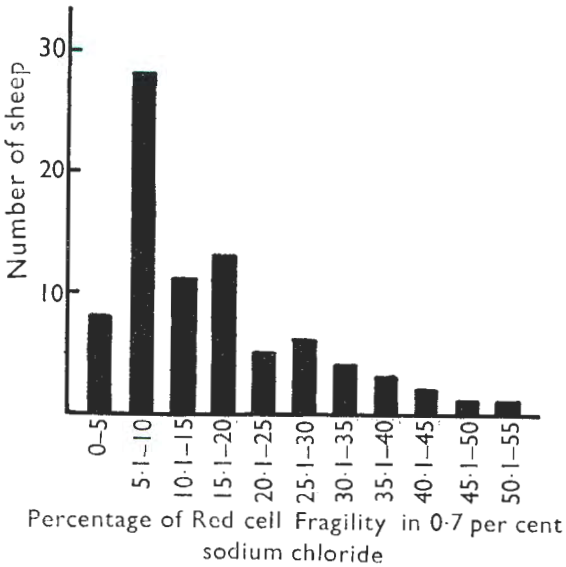

FIG. 2b.—Red cell fragility test—Group 2.

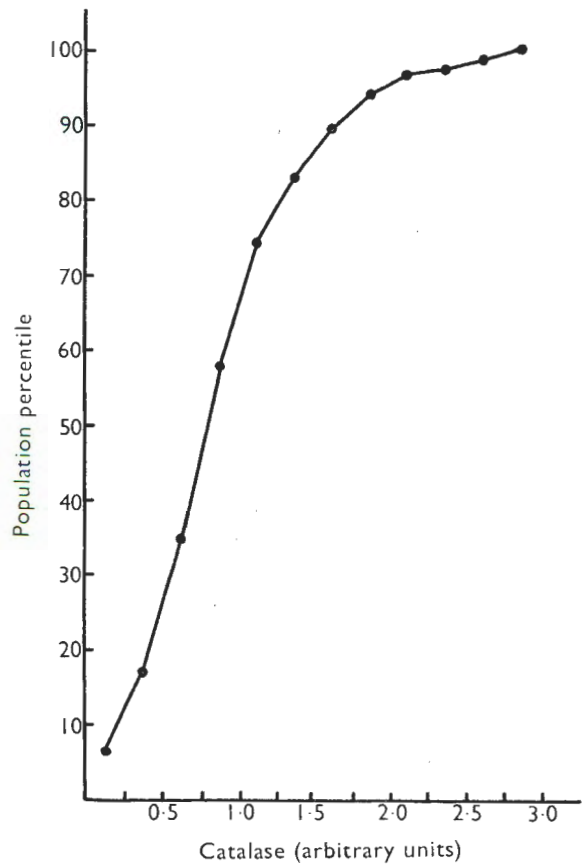

FIG. 3.—Plasma catalase—Group 1.

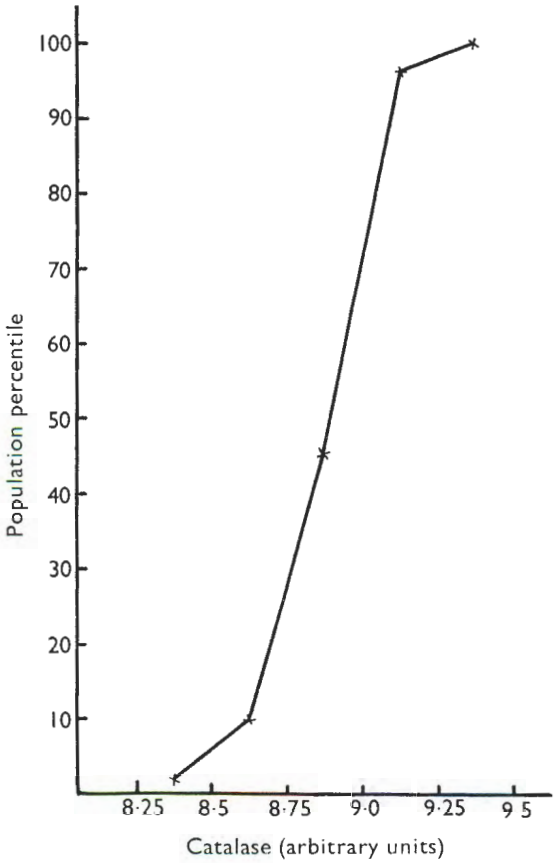

FIG. 3a.—Plasma catalase—Group 2.

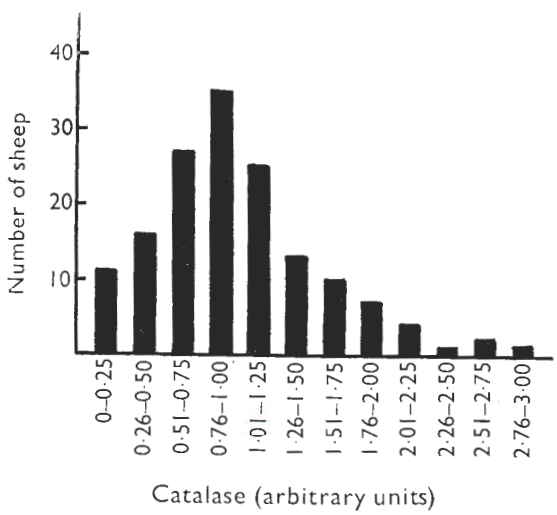

FIG. 3b.—Plasma catalase—Group 1.

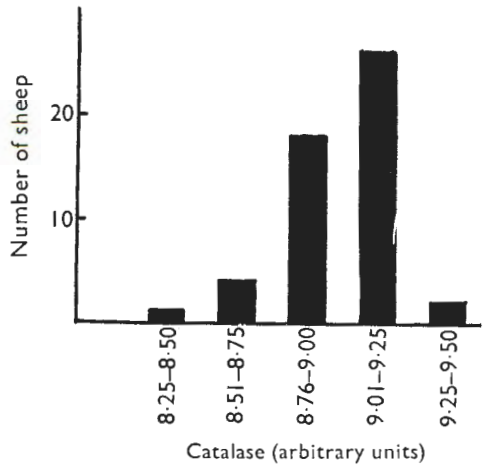

FIG .3c.—Plasma catalase—Group 2.

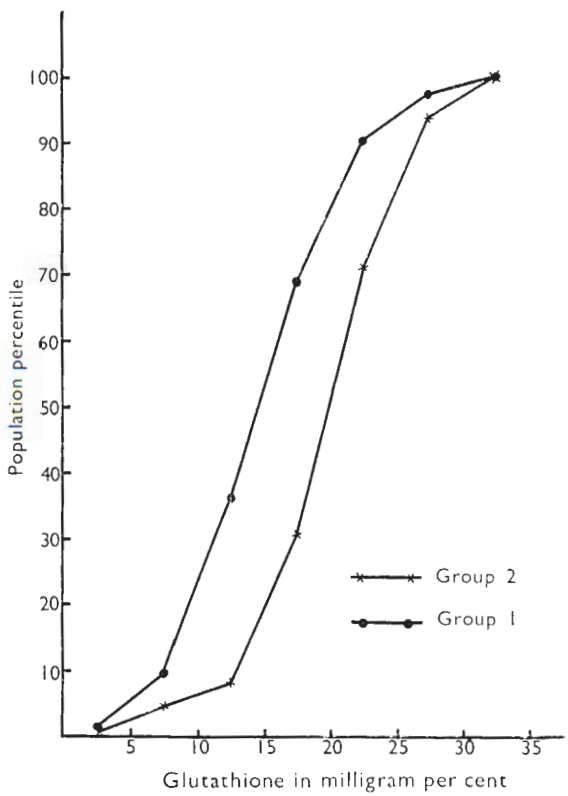

FIG. 4.—Blood glutathione.

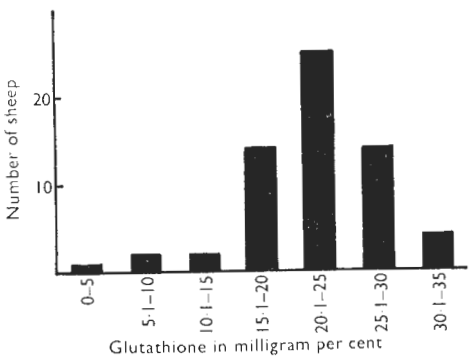

FIG. 4a.—Blood glutathione—Group 1.

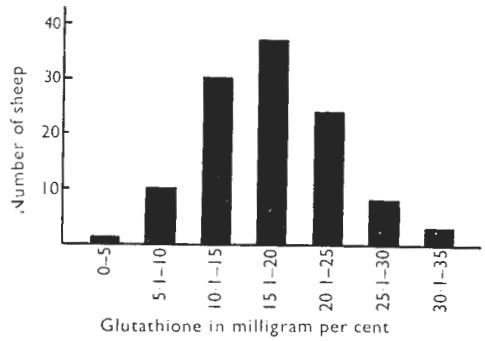

FIG. 4b.—Blood glutathione—Group 2.

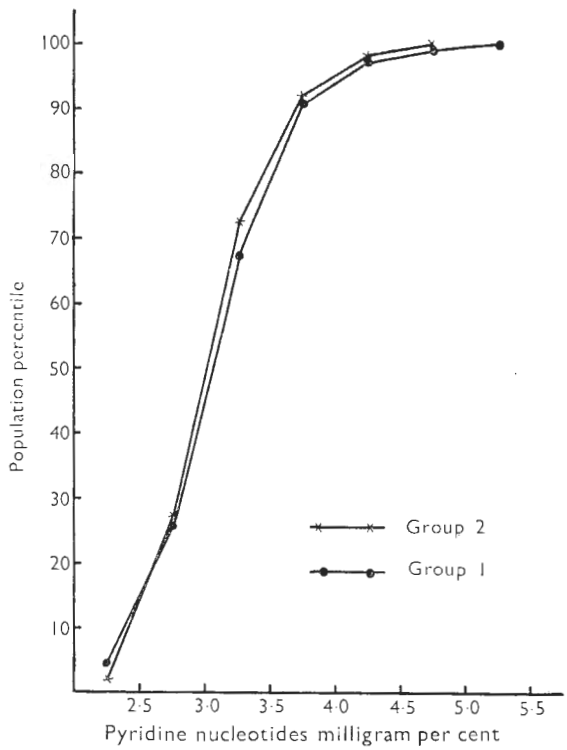

FIG. 5.—Blood pyridine nucleotides.

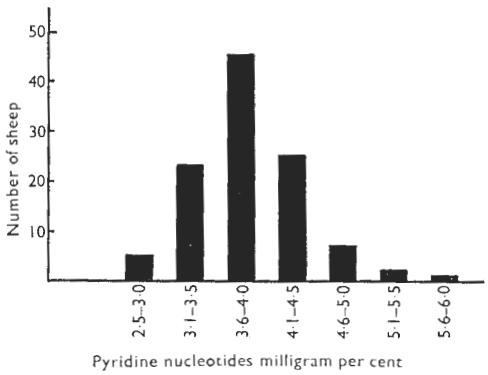

FIG. 5a.—Blood pyridine nucleotides—Group 1.

FIG. 5b.—Blood pyridine nucleotides—Group 2.