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# Methaemoglobin and erythrocyte reducing systems in high-altitude natives

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**Summary.** The stress of chronic hypobaric hypoxia present at high altitudes induces a series of adaptive changes in the intermediate metabolism in erythrocytes of high-altitude natives. Aymaras of the high Andean Plateau are shown to have within erythrocytes: (*a*) increased activity of $NADH_2$ (GAPDH) generating stages, (*b*) decreased activity of $NADH_2$ (LDH) consuming steps, (*c*) significantly increased methaemoglobin content, and (*d*) a large increase in the level of reduced glutathione. These alterations occur also in persons of the same ethnic group residing at low altitude. There is, however, only a moderate elevation of classic haematological parameters (erythrocyte count, haemoglobin and haematocrit) in highland natives. The functional implications of these metabolic changes are discussed with respect to regulation of erythrocyte metabolism.

List of abbreviations

| | |
|---|---|
| $NADH_2$ | Nicotinamide-adenine dinucleotide, reduced form |
| $NADPH_2$ | Nicotinamide-adenine dinucleotide phosphate, reduced form |
| NAD | Nicotinamide-adenine dinucleotide, oxidized form |
| GAPDH | Glyceraldehyde-3-phosphate dehydrogenase |
| LDH | Lactate dehydrogenase |
| G6PD | Glucose 6-phosphate dehydrogenase |
| 6PGD | 6-Phosphogluconate dehydrogenase |
| GSH | Reduced glutathione |
| GS-SG | Oxidized glutathione |
| $pO_2$ | Partial oxygen pressure |
| WB | Whole blood |
| MB | Methylene blue |
| Met Hb | Methaemoglobin |

## 1. Introduction

One of the greatest physiological problems the circulating red blood cell has during its 120-day life cycle is in maintaining haemoglobin function and respiratory capacity. The circulating erythrocyte contains a series of precise and efficient mechanisms utilizing reduced cofactors formed at various stages of glycolysis (Kaplan 1966, Scott, Duncan and Ekstrand 1965) which ensure this. Our knowledge of these processes, however, comes mainly from studies on populations residing at or near sea level and little is known about how adaptation to chronic hypoxia alters these systems.

The aim of this investigation is to analyse the behaviour of these systems in erythrocytes from a sample group of highland natives who have been living for many generations at high altitudes (>3000 m) where they are exposed to the chronic environmental stress of hypobaric hypoxia. They may be presumed to have undergone appropriate adaptive changes (Ruffié, Vergnes and Hobbe 1966, Vergnes and Larrouy 1967). We have compared a group of Amerindians of the Bolivian Altiplano to a similar group of the same ethnic background who have migrated to lower altitude.

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## 2. Materials and methods

Two groups of Aymara males born in the Bolivian Altiplano (Department of La Paz) were studied in the communities where they lived. Group A consisted of 85 adults (age 22 ± 3 years) who were born and have always lived in La Paz (altitude 3600 metres above sea level). Group B contained 84 adults (age 23 ± 7 years) born at an altitude of approximately 3600 metres and living for more than three years at Santa Cruz (altitude 400 metres). These Aymaras may be considered to have passed the two main stages of acclimatization (six months) and of physiological adaptation (one to two years) to their new environment (Baker and Little 1976).

Blood samples were obtained by venepuncture from resting subjects and were collected with appropriate anticoagulants. These specimens were analysed in the communities where they were collected (La Paz and Santa Cruz) and they were not subjected to transportation. The laboratory material and techniques were the same in both cases.

Haematological parameters of erythrocyte count, haematocrit and haemoglobin concentration were obtained on each individual by standard methods.

Enzymatic reducing systems were analysed by the following procedures:

(*a*) methaemoglobin concentration by the spectrophotometric method of Evelyn and Malloy (1938);
(*b*) methaemoglobin conversion by the method of Brewer, Tarlov and Alving (1960) in the presence of glucose with and without methylene blue (incubation was carried out at 37°C with constant mixing);
(*c*) reduced glutathione in erythrocytes by the method of Kaplan and Dreyfus (1964) with 5,5′-dithiobis-2-nitrobenzoic acid;
(*d*) reduced glutathione instability in the presence of acetyl phenylhydrazine by the method of Beutler (1957);
(*e*) reduced glutathione instability by the method of Mills and Randall (1958);
(*f*) GAPDH, LDH and G6PD activity by the protocol of Cartier, Leroux and Marchand (1967);
(*g*) $NADH_2$ diaphorase activity by the method of Hegesh, Calmanovici and Avron (1968), and $NADPH_2$ diaphorase activity by the method of Sass, Caruso and Farhangi (1967);
(*h*) glutathione reductase activity by the method of Horn (1973).

All statistical comparisons of groups were made using Student's *t* test.

## 3. Results

The data are displayed in two tables. Table 1 shows haematological and biochemical parameters obtained in the two groups. Table 2 gives comparative enzymatic activities in the two groups. Statistically significant differences are indicated.

## 4. Discussion

Quantitative differences in the haematological variables (table 1) of haemoglobin, haematocrit and erythrocyte counts are seen in Aymaras living at high altitude, as compared with those who migrated to the low-altitude area of Santa Cruz.

There are some unusual and complex changes occurring in the erythrocyte enzyme systems of the highland residents which are illustrated in table 2. It has previously been reported (Arnaud, Vergnes and Gutierrez 1976 a) that rate constants for key glycolytic enzymes in erythrocytes of high-altitude natives are significantly altered and indicate some changes in flux in the intermediate oxidation–reduction systems.

Table 1. Data derived from the study of erythrocyte reduction systems in two Aymara groups living at two different altitudes.

| | | | | | | Brewer's test for MetHb conversion in % | | | Instability test of GSH | | Mills and Randall's Test of GSH | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Ht (%) | Hb (g/100 ml WB) | Number of red cells ($10^6/mm^3$) | MetHB (%) | With M.B. | Without M.B. | GSH (mg/100 ml red cells) | (mg/100 ml red cells) | (%) | (mg/100 ml red cells) | (%) |
| Group A | $n$ | 85 | 85 | 85 | 85 | 85 | 85 | 85 | 85 | 85 | 85 | 85 |
| from | $\bar{x}$ | 51·75 | 18·23 | 5·74 | 3·14 | 7·79 | 47·31 | 79·84 | 68·89 | 86·27 | 70·25 | 88·34 |
| La Paz (3600 m) | S.D. | 2·93 | 1·09 | 0·33 | 0·89 | 1·50 | 8·66 | 8·49 | 10·14 | 8·44 | 10·03 | 7·29 |
| Group B | $n$ | 84 | 84 | 84 | 84 | 81 | 81 | 84 | 83 | 83 | 84 | 84 |
| from | $\bar{x}$ | 41·20 | 14·54 | 4·40 | 1·52 | 3·80 | 39·00 | 72·64 | 61·28 | 84·49 | 63·48 | 87·13 |
| Santa Cruz (400 m) | S.D. | 4·12 | 1·42 | 0·48 | 0·63 | 1·14 | 7·54 | 10·20 | 11·30 | 9·29 | 10·88 | 8·07 |
| Statistical | $t$ | 8·54 | 7·96 | 8·29 | 12·15 | 10·20 | 3·18 | 2·98 | 2·60 | 1·69 | 2·65 | 1·73 |
| analysis | $P$ | <0·001 | <0·001 | <0·001 | <0·001 | <0·001 | <0·01 | <0·01 | <0·01 | <0·1 | <0·01 | <0·1 |

Table 2. Comparative study of the enzymes of the erythrocyte reduction systems in two Aymara groups living at two different altitudes.

| | | GAPDH† | LDH† | G6PD† | $NADH_2$ diaphorase‡ | $NADPH_2$ diaphorase‡ | GSSG reductase† |
|---|---|---|---|---|---|---|---|
| Group A High altitude (3600 m) | $n$ | 50 | 50 | 50 | 50 | 50 | 50 |
| | $\bar{x}$ | 19·88 | 18·96 | 1·490 | 0·929 | 1·676 | 0·685 |
| | S.D. | 1·51 | 1·27 | 0·176 | 0·193 | 0·383 | 0·106 |
| Group B Lowland (400 ml) | $n$ | 30 | 30 | 30 | 30 | 30 | 30 |
| | $\bar{x}$ | 14·70 | 22·27 | 1·570 | 0·940 | 3·000 | 0·596 |
| | S.D. | 3·41 | 4·27 | 0·240 | 0·110 | 1·100 | 0·120 |
| Statistical analysis | $t$ | 2·98 | 2·38 | 0·68 | 0·57 | 7·58 | 2·41 |
| | $P$ | <0·01 | <0·05 | <0·1 | <0·1 | <0·001 | <0·02 |

† in I.U./ml red cells.
‡ in I.U./g Hb.

Glyceraldehyde phosphate dehydrogenase (GAPDH) activity is increased by approximately 35%, while lactate dehydrogenase (LDH) activity is decreased by 25% in group A relative to group B. These relative changes in activity of $NADH_2$ generation (GAPDH) versus $NADH_2$ consumption (LDH catalysing the pyruvate→lactate reaction) consequently leads to an elevation of the $NADH_2$/NAD ratio. The two dehydrogenases of the pentose pathway (G6PD and 6PGD) also seem to supply large quantities of reducing equivalents as shown by the elevated levels of reduced glutathione in the highland residents (table 1) as well as increased activities of these two enzymes (table 2). It has previously been shown (Arnaud, Vergnes and Gutierrez 1976 b) in genetically similar groups that increased glucose consumption at high altitude favours increased pyridine nucleotide reduction in both glycolytic and pentose pathways. From the elevated $NADH_2$/NAD ratio and the increased quantities of GSH, it is possible to postulate that a sufficient supply of reducing equivalents exists for utilization in erythrocyte systems (Eifler and Wegenknecht 1964).

Paradoxically, we find these same individuals have an elevated methaemoglobin level (table 1). A plausible explanation is the relative hypoactivity of $NADH_2$ in the reduced state. Incubation tests for methaemoglobin reduction in the presence of methylene blue, however, do not show a preferential utilization of $NADPH_2$ diaphorase for haemoglobin reduction in Aymara living at high altitude. These findings confirm data presented by Stromme and Eldjarn (1962) using direct determination of enzyme activity.

Indirect methods for measuring $NADH_2$ diaphorase activity (Brewer *et al.* 1960) without methylene blue confirm the trends of a decrease in activity in high-altitude Aymara as determined by direct activity methods (tables 1 and 2); this is coincident with the significant elevation of residual methaemoglobin.

This depression in $NADH_2$ diaphorase parallels a decrease in $NADPH_2$ diaphorase activity, as shown by an indirect technique, and hence it is tempting to suggest a similar sensitivity and response to environmental hypoxia even though NADH-dependent diaphorase is known to be much more physiologically important.

We only made direct measurement of $NADPH_2$ diaphorase on 50 Aymaras in La Paz (group A). The average value was $0{\cdot}470 \pm 0{\cdot}103$ IU/g Hb. We have no results for this enzyme measurement in group B, so no comparison is possible. But the average value found in a North American population living at sea level is $2{\cdot}780 \pm 0{\cdot}740$ IU/g Hb (Sass *et al.* 1967).

Intra-erythrocyte concentrations of reduced glutathione are increased at high altitude and this confirms results previously reported by Delrue, Vischer and Bouckaert (1933). It is possible that these increased amounts of reduced tripeptide are the result of increased activity of glutathione reductase itself or alternatively of increased levels of substrate $NADPH_2$ imposed by glycolytic hyperactivity (Arnaud *et al.* 1976 b). Possible biological implications of this elevation in reduced glutathione levels are:

(1) an increased utilization of the tripeptide in methaemoglobin reduction (Kaplan 1969). Glutathione levels may play a regulatory role in governing methaemoglobin reduction rate or may also function, as Horejsi (1970) has suggested, by a regulatory role in oxygen fixation by haemoglobin. Alternatively there might be;

(2) an increased requirement in the erythrocyte glutathione peroxidase system for the reduced substrate, glutathione. Unfortunately, it is not possible at this time to ascertain the reasons for the elevation of glutathione in highland Aymara.

Finally, there is the interesting relationship between methaemoglobin levels and general regulation of erythrocyte carbohydrate metabolism. Murphy (1960) and Minakami (1969) suggested the fundamental role of oxygen as a regulator of glycolytic sequences. Reduced pyridine nucleotides formed during the degradation stages of glycolysis are seen to be elevated in Aymara living at high altitude and this elevation is supported by the data on glycolytic and pentose pathway enzyme activity.

One may consider then, that methaemoglobin in these high-altitude residents constitutes a non-functional, but easily mobilized, mass of haemoglobin that could displace the oxygen–haemoglobin dissociation curve (Darling and Roughton 1942, Gourdin, Vergnes and Gutierrez 1975) or be rapidly transformed into active reduced haemoglobin according to the needs of the system. This transformation would probably occur through the combined action of the diaphorase enzyme systems and reduced glutathione levels. The concept of a haemoglobin reserve in a non-functional state is not well expressed when amounts of methaemoglobin are reported as percentages of total haemoglobin. Values expressed as grams per 100 ml of whole blood are much more meaningful. In lowland Aymara, 1·52% MetHb in a total haemoglobin concentration of 14·54 g/100 ml blood represents a concentration of methaemoglobin of 0·22 g/100 ml whole blood. Highland Aymara, with 3·14% MetHb in a total concentration of 18·23 g/of blood haemoglobin, have a concentration of haemoglobin equal to 0·57 g/100 ml whole blood. A ratio of the MetHb percentages for comparison of high altitude to low equals 2·07, while the ratio expressed in concentration equals 2·59.

In considering internal mechanisms of MetHb levels, one may postulate either a direct effect of oxygen on enzymatic machinery or the consequence of intracellular pH changes. The latter is more likely when one looks at experimental data of others (Minakami 1969, Arnaud, Gutierrez and Vergnes 1977) on $pO_2$ and acid–base balance.

## 5. Conclusions

(1) Amerindians who have lived for many generations in an environment of chronic hypobaric hypoxia have modifications of erythrocyte metabolism such as elevated MetHb levels. These alterations probably are indicative of complex adaptive processes occurring at many levels.

(2) The biochemical and haematological adjustments represent the effects of the environment on intermediary metabolism and its regulation in man. These adaptations acquired under the persistent physiological constraint produced at high altitude may be considered as functional adaptations if they can be related to individual survival. Such processes may be a direct result of gene–environment interaction, since this unique environment may exert a very strong selective pressure on the living organism. Analysis of the adaptive responses of highland populations offer an excellent opportunity for study in this new area of geographic haematology.

## References

ARNAUD, J., VERGNES, H., and GUTIERREZ, N., 1976 a, Respiratory function and erythrocyte metabolism at high altitude. *Hematologia* (*Hematology*), **6**, 73–87.

ARNAUD, J., VERGNES, H., and GUTIERREZ, N., 1976 b, Fonction respiratoire et métabolismes érythrocytaires en haute altitude. Les Colloques de l'INSERM—Anthropologie des Populations Andines. *INSERM* **63**, 505–522.

ARNAUD, J., GUTIERREZ, N., and VERGNES, H., 1977, Funcion respiratoria del globulo rojo en la altura. *Sangre*, **22**, 219–224.

BAKER, P. T., and LITTLE, M. A., 1976, *Man in the Andes. A Multidisciplinary Study of High Altitude Quechua* (Stroudsburg, Pa.: Dowden Hutchinson and Ross).

BEUTLER, E., 1957, The glutathione instability of drug sensitive red cells. A new method for the *in vivo* detection of drug sensitivity, *Journal of Laboratory and Clinical Medicine*, **49,** 84–95.

BREWER, G. J., TARLOV, A. R., and ALVING, A. S., 1960, Methaemoglobin reduction test. A new simple *in vitro* test for identifying primaquine sensitivity. *Bulletin of the World Health Organization*, **22,** 633–639.

CARTIER, P., LEROUX, J. P., and MARCHAND, J. C., 1967, Techniques de dosage des enzymes glycolytiques tissulaires. *Annales de Biologie Clinique*, **25,** 109–136.

DARLING, R. D., and ROUGHTON, F. J. W., 1942, The effect of methemoglobin on the equilibrium between oxygen and hemoglobin. *American Journal of Physiology*, **137,** 56–68.

DELRUE, G., and VISCHER, A., 1933, Modification du taux de glutathion sanguin durant le séjour à haute altitude. *Compte Rendu des Séances de la Société de Biologie*, **113,** 942–944.

EIFLER, R., and WEGENKNECHT, C., 1964, $NADH_2$ und $NADPH_2$ specifische GSSG Reduktasen in roten Blutzellen, *Acta Medica et Biologica* **13,** 674–682.

EVELYN, K. A., and MALLOY, H. T., 1938, Microdetermination of oxyhemoglobin, methemoglobin and sulphemoglobin in a single sample of blood. *Journal of Biological Chemistry*, **126,** 665–662

GOURDIN, D., VERGNES, H., and GUTIERREZ, N., 1975, Methaemoglobin in man living at high altitude. *British Journal of Haematology*, **29,** 243–246.

HEGESH, E., CALMANOVICI, N., and AVRON, M., 1968, New method for determining ferrihemoglobin reductase (NADH-methemoglobin reductase) in erythrocytes. *Journal of Laboratory and Clinical Medicine*, **72,** 339–344.

HORN, H. D., 1973, *Glutathione Reductase in Methods Enzymatic Analysis* (New York: Academic Press).

HOREJSI, J., 1970, Effect of glutathione and some other substances on the oxygen dissociation curve of hemoglobin and experimental therapy of hemorhagic shock with solutions enriched with glutathione. In *Red Cell Metabolism and Function. Advances in Experimental Medicine and Biology*, edited by G. J. Brewer, vol. 6, pp. 9–15.

KAPLAN, J. C., and DREYFUS, J. C., 1964, Dosage du glutathion érythrocytaire par un disulfure aromatique nitré l'acide 5,5'-dithiobis-(2-nitrobenzoique). *Bulletin de la Société de Chimie Biologique*, **46,** 775–783.

KAPLAN, J. C., 1966, Les systèmes d'oxydo-réduction du globule rouge et leurs anomalies. *Nouvelle Revue Française d'Hématologie*, **6,** 809–834.

KAPLAN, J. C., 1969, Diaphorases et glutathion réductase érythrocytaires. *Exposés Annuels de Biochimie Médicale* (Paris: Masson édit.), vol. 29, pp. 77–99.

MILLS, G. C., and RANDALL, H. P., 1958, Hemoglobin catabolism. II. The protection of hemoglobin from oxidative breakdown in the intact erythrocyte. *Journal of Biological Chemistry*, **232,** 589–598.

MINAKAMI, S., 1969, Effect of oxygen tension on glycolysis in erythrocyte. *Fürsvarsmedicin*, **5,** 181–189.

MURPHY, J. R., 1960, Erythrocyte metabolism. II. Glucose metabolism and pathways. *Journal of Laboratory and Clinical Medicine*, **55,** 286–302.

RUFFIÉ, J., VERGNES, H., and HOBBE, TH, 1966, Sur la réversibilité de la methémoglobinisation des hématies chez les populations indigènes du corridor interandin. Essai d'interprétation. *Compte Rendu de l'Académie des Sciences*, **262,** 1956–1958.

SASS, M. D., CARUSO, C. J., and FARHANGI, M., 1967, TPNH-methemoglobin reductase deficiency: a new red-cell enzyme defect. *Journal of Laboratory and Clinical Medicine*, **70,** 760–767.

SCOTT, E. M., DUNCAN, I. W., and EKSTRAND, V., 1965, The reduced pyridine nucleotide dehydrogenases of human erythrocytes. *Journal of Biological Chemistry*, **240,** 481–485.

STROMME, J. H., and ELDJARN, L., 1962, The role of the pentose phosphate pathway in the reduction of methaemoglobin in human erythrocytes, *Biochemical Journal*, **84,** 406–410.

VERGNES, H., and LARROUY, G., 1967, Les déficits en G6PD dans les populations des Andes Boliviennes. *Nouvelle Revue Française d'Hématologie*, **7,** 124–128.

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**Zusammenfassung.** Die Belastung durch chronische hypobarische Hypoxie in großer Höhe induziert eine Reihe von adaptiven Veränderungen des intermediären Metabolismus der Erythrozyten bei dort lebenden Eingeborenen. Bei den Aymara des Hochplateaus der Anden zeigt sich in den Erythrozyten: (*a*) erhöhte Aktivität der Synthesestufen von $NADH_2$ (GAPDH), (*b*) herabgesetzte Aktivität der Verbrauchsstufen von $NADH_2$ (LDH), (*c*) signifikant erhöhter Anteil von Methämoglobin, und (*d*) ein wesentlicher Anstieg im Niveau des reduzierten Glutathion. Diese Veränderungen kommen auch bei Personen derselben ethnischen Gruppe vor, die in niedriger Höhenlage wohnen. Bei den Eingeborenen des Hochlands gibt es jedoch nur eine bescheidene Erhöhung der klassischen hämatologischen Parameter (Erythrozytenzählung, Hämoglobin und Hämatokrit). Die funktionale Bedeutung dieser metabolischen Veränderungen werden diskutiert vor dem Hintergrund der Regulation des Erythrozytenmetabolismus.

**Résumé.** Le stress de l'hypoxie hypobare présent aux hautes altitudes induit une série de changements adaptatifs dans le métabolisme intermédiaire des érythrocytes des indigènes de haute altitude. Les Aymara du haut Plateau Andin présentent dans leurs érythrocytes: (*a*) une activité accrue des stades générateurs de

$NADH_2$ (GAPDH), (*b*) une activité réduite des étapes de consommation de $NADH_2$ (LDH), (*c*) un taux significativement accru de méthémoglobine, et (*d*) une augmentation importante du taux de glutathion réduit. Ces modifications s'observant aussi chex les personnes de même groupe ethnique résidant à basse altitude. Il n'y a cependant qu'une élévation modérée des paramètres hématologiques classiques (compte érythrocytaire, hémoglobine et hématocrite) chez les indigènes d'altitude. Les implications fonctionnelles de ces changements métaboliques sont discutées par rapport à la régulation du métabolisme des érythrocytes.