Hiroshima Journal of Medical Sciences
Vol. 32, No. 1, March, 1983
HIJM 32-19

Determination of Amino Acids and Polyamines in Human Erythrocytes
Part 1. Fundamental Studies*)

Akira TOKUNAGA

Second Department of Surgery, Hiroshima University School of Medicine, Hiroshima 734, Japan
(Director: Prof. Haruo EZAKI)

(Received December 25, 1982)

Key words: Amino acid, Polyamine, Erythrocyte

ABSTRACT

1) A simple, rapid analytical method for amino acids and polyamines in erythrocytes within a blood volume of 1 ml with a quantitative limit of precision ranging from 1-10 p moles was established using high performance liquid chromatography (HPLC)
2) A capillary sampling system (C-S system) which consists of a propylene capillary tube for hematocrit measurement was adopted for treatment of the erythrocytes. After fillihg the tube with blood, it was treated with a variety of method.
3) The free amino acids in erythrocytes had such characteristics as (1) high levels of acidic amino acids, (2) low levels of Val, Met, Ile, Phe, Trp and Arg and (3) branched chain amino acids (Val, Ile, Leu) had a tendency to readily pass through the erythrocyte membrane.
4) Washing of erythrocyte pellets with saline was not good for measurement of amino acids in erythrocytes, particularly the branched chain amino acids. The space rate of non-washed erythrocyte pellets was calculated as (free amino acid levels in blood pellets − space rate × free amino acid levels in plasma).
5) Polyamine level in erythrocytes changes in accordance with aging of erythrocytes, i. e. it begins decreasing from the time reticulocytes are released from the bone marrow and continues throughout the course of mature erythrocytes up to the time of destruction in the spleen.

INTRODUCTION

Generally, surgical invasion produces bleeding, dehydration, destruction of local tissue, increase in metabolites, effects of anesthesia, infection, dietary restriction, need for bedrest etc., which result in differences in the postoperative condition[36,42]. These in turn, cause changes in water, electrolytes, carbohydrates, fat and protein metabolism, especially, pre- and postoperative protein metabolism, among which the free amino acids in plasma are one of the major factors that affect the postoperative course[23,24,27,30,43]. Recently, the use of enteral- and intravenous transfusion of high calory amino acids has increased, thus drawing much attention[15,40].

On the other hand, there are yet many obscure points about the physiological function of polyamines, but many reports[7,17,22,34] have indicated that it is closedly involved in cell proliferation and nucleic acid metabolism. Their usefulness in daily clinical examination has been recognized since Russell et al.[35] (1971) reported their elevation in urine of cancer patients, and subsequently attention has been focussed upon them as markers for the diagnosis of cancer. Lately, there are reports of polyamine determination being made on urine[48], serum[31,46], cerebrospinal fluid (csf)[26,49], erythrocytes[6,8,9,13,14,37,41,44,45,47,50-52], biopsy specimens[1,2,11,28,29,32,33,53], etc... Erythrocytes are readily available in the clinic, and their intracellular constituents have been almost completely elucidated[5,21], but

*) 徳永　彰：人赤血球中のアミノ酸・ポリアミン測定　第1 基礎編

there is a paucity of reports on the components in the pathological states[38]. The function of erythrocytes consists of transportation of O_2 & CO_2 by hemoglobins and production of energy necessary for autometabolism. With the purpose of elucidating the erythrocyte constituents, especially the changes in polyamines which are components of protein and are involved in the synthesis of amino acids and proteins that, in turn, participate in protein metabolism, we focussed our attention on the capillary sampling system (C-S system) of Sasaki et al.[39]. This method is considered to be an epoch-making procedure for routine determination of intracellular material, and can also be used for hematocrit measurement. The application of this method, proved to be a rapid and highly sensitive procedure based on HPLC using simple erythrocyte treatment for measuring free amino acids and polyamines in erythrocytes. Study was made to determine the changes in free amino acids and polyamines in erythrocytes during the pre- and post-surgical operation stages, the implication of such changes and whether the findings can be used in routine clinical examinations.

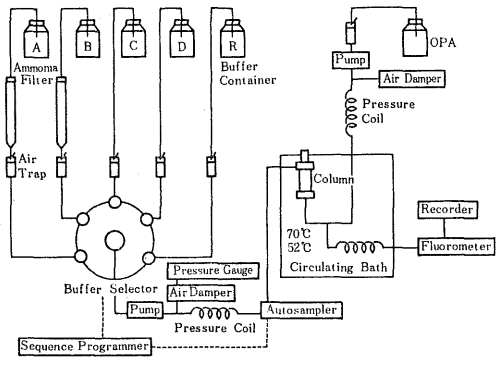
MATERIAL AND METHODS

1) Material
Blood was drawn early in the morning in fasting state, and was restricted, as a rule, to males whose ages ranged from 20 to 40 years.

2) High Performance Liquid Chromatography (HPLC, Fig. 1) The analytical apparatus used for amino acids and polyamines was a self-made unit developed by Yoshida[20], [54-56] et al.. Three types of stepwise isocratic elutions were used, i. e.
(A) 0. 2N Na citrate buffer, pH 3. 25 containing 8% n-propanol
(B) 0. 2N Na citrate buffer, pH 4. 25 and
(C) 0. 9N Na citrate buffer, pH 9. 4
Elution buffers (A) and (B) were refined by use of an ammonia filter. Solutions (A), (B), (C) & (R) were put into an auto-solvent changer (Kyowa Seimitsu KK) to combine the solution into one. The pulsation of the stream of buffer elution from the minimicropump (plunger type, Kyowa Seimitsu KK) was eliminated with an air damper and pressure gauge, and was led into the auto-sampler and sample injector (Rheodyne), and then into the column of a constant temperature water bath. The column contained such cation exchange resins as TSK, GEL, IEX 215 (Toyo Soda Co Ltd., Tokyo). The particle sizes were 8-10μl, and the column size was 0. 4 cm (ϕ) × 30. 0 cm. This is known as the post-label method. Orthophthalaldehyde (OPA) solution was delivered similarly through a minimicropump and mixed using a triple headed joint. The flow rate for mixing was 1 : 1. The reaction fluid was sent to the fluorometer (Shimazu Seisakusho FLD-1) through the reaction coil (diameter 0. 5 mm). A fluorescence intensity at 455 nm was recorded with a recorder (Yokogawa Denki KK, Type 3066) at 350 nm excitation. As soon as each detection was completed, the column was regenerated using regenerating fluid. the analytical time required was about 60 min with a quantitative limit of 1-10 pico mole (p mol).

The elution buffer for polyamines was 2. 0N Na citrate buffer containing 20% metanol (pH 5. 28). The cation exchange resins of TSK, GEL, IEX 215 were packed in columns of the size of 0. 4 cm (ϕ) × 8. 0 cm and kept at a temperature of 70°C. As internal standard material, triethylenetetramine [2·2·2]N_4·4HCl (Wako Junyaku) was used. The analytical time required for polyamine was about 20 min.

3) Preparation of human plasma (Table 1)
About 1 ml of blood was drawn from the cubital vein early in the morning in fasting state, and flushed into a heparinized spitz



Schematic illustration of analytical system

Fig. 1. Schematic illustration of analytical system

Table 1. Sample preparation of human erythrocytes

Human Blood (Ca 1ml)
Centrifugation, 1600×G, 5min, 20℃
Supernate — Cell pellet
Deproteination with 10% TCA — Washing with 10ml of saline (3 times)
Amino Acid Analysis (I. S: Nle) — Cell pellet — Supernate
1) Shaking
2) Preparation of red cell pellet (According to the Method of Sasaki et al.)
3) Centrifugation 13000×G, 20min, 4℃
Cut capillary tube (2cm-60μl)
1) Destruction and Deproteination of Red Cells with 300μl of solution containing 1% Triton, 10% TCA, [2·2·2]N_4 and Nle.
2) Shaking & mixing
3) Centrifugation 1600×G, 5min, 20℃
Supernate 100ml — Residue
Amino Acid Analysis (I. S: Nle.)

tube. This was centrifuged (1600×G, 5 min) at room temperature, and separated into plasma and cell components. A 100 μl volume of the plasma component was shake-mixed with 1 ml of 10% trichloracetic acid (TCA) solution (Norleucine: Nle internal standard solution 10^{-5}M). After deproteinization, it was centrifuged (1600×G, 5 min), and the supernatant was used as sample for amino acid analysis.

4) Preparation of human erythrocytes (Table 1)

The buffy coat layer was removed from the remaining cell component with a pipette, and the cell component was shake-mixed with a thermomixer for one minute. The blood cell component was aspirated with a 1 ml syringe, and quickly packed into polypropylene capillary tubes (Nipro Iko Co., inner diameter 0.2 cm, length 6.8 cm) (Fig. 2). The packed tubes were plugged at one end with clay (crytoseal for hematocrit measurement), and gunshot (shotgun size No. 8). These were placed in a specially order-made rotor (it has wider grooves than the rotor for hematocrit measurement) (Photo. 1). The tubes were spun using a

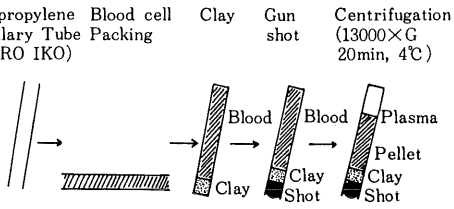


Fig. 2. Preparation of blood cell pellet

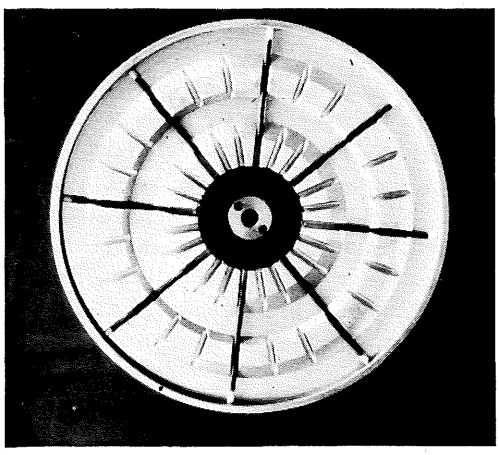


Photo. 1. Rotor with polypropylene capillary tube

refrigerated high speed centrifuge (Kubota KR 180 B type, 13000×G, 20 min, 4°C). After centrifugation, sections 2 cm in length of the tubes were cut off with a safety razor from the mild lower portion (2 cm to 4 cm from the bottom) (Photo. 2). One end of the capillary tube was connected to a fractionator (Socolex Dispensor 10), and the cell pellet was flushed out into a spitz test tube using 10 ml of 0.9% physiological saline. The tube was shake-mixed and centrifuged (1600×G, 3 min, 20°C), after which the supernatant was drained. This procedure was repeated twice to wash the

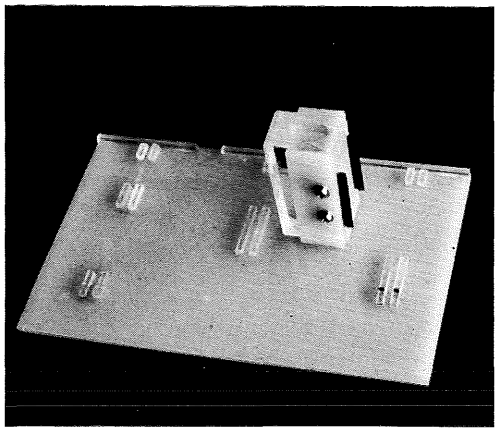
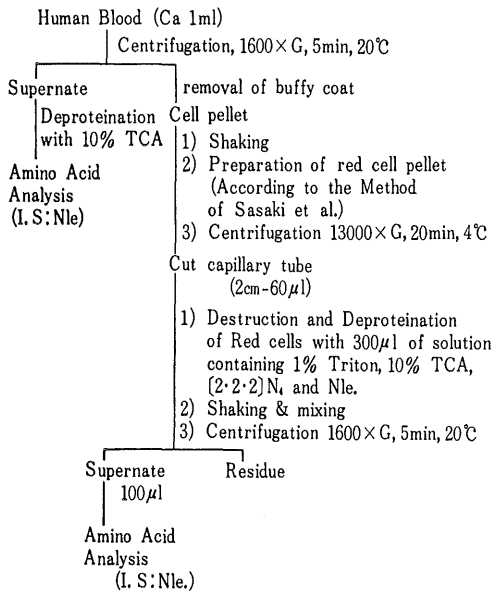


Photo. 2. Board & Cutter

erythrocyte component. The pellet was immersed in 10% TCA and 1% Triton X-100D solutions combined together in equal volumes of 0.15 ml to which Nle (2×10^{-5}M) was added to destroy the erythrocyte membrane and carry out deproteinization. This solution was centrifuged at $1600\times G$ for 3 min, 20°C and 0.1 ml of the supernatant was diluted down 5-fold for use as test material for analysis of amino acids in erythrocytes. Two capillary tubes were prepared by the aforesaid method. the lower end of 2 tubes were cut at 1 cm, and 2 tubes at 5-6 cm from the bottom. These were immersed in 10% TCA and 1% Triton X-100D combined together in equal volumes of 0.15 ml and Nle (2×10^{-5}M) to make a total solution of 0.3 ml. The container was shaken gently to elute the contents which were then subjected to deproteinization. After centrifugation, 0.12 ml of supernatant was obtained. This was lyophilized and concentrated to 4-fold for use as test material for polyamine analysis (Table 2).

Table 2. Sample preparation of human erythrocytes



5) Preserving of whole blood
It is best if the cells can be treated immediately after collection, but when this is not possible it necessitates preservation of blood. Thus, study was made on such a method to assertain the length of time of preservation. The volume of free amino acids in plasma and erythrocytes were determined at room temperature (20°C) and refrigeration (4°C) at the four-time periods of 1, 3, 9, 27 hr after collection.

6) Effects of number of washings
Plasma components were still present in the spaces between erythrocytes after refrigerated high speed centrifugation. According to estimates made by M. Sasaki[39], they amounted to 2.4%. In order to eliminate the effects of such plasma components after washing, a study was made to determine the extent of their effects on free amino acids and polyamines in erythrocytes. Using 1 ml of whole blood, comparisons were made without washing and with washing using 10 ml of 0.9% physiological saline 1, 3, 5 and 7 times.

7) Permeability of erythrocyte membrane
There are no extramembranous amino acids during washing, but it was considered necessary to study the effects of such intravenous amino acids when the outside concentration increases following routine clinical transfusion. To 2 ml of whole blood was added 12% Ispol solution (EAA : NEAA=1 : 1, Daigo Eiyo Co. Ltd.) in volumes of 30, 90, 180 μl (equivalent to ×8, ×23 and ×46 of human plasma), and after refrigeration for 1 hr, the amino acid volumes in plasma and erythrocytes were determined. Assuming that the polyamine volumes in erythrocytes were Putrescine (Put) 2034 nano mole/ml (n mol/ml), Spermidine (Spd) 6.71 n mol/ml and Spermine (Spm) 3.49 n mol/ml, 5×, 25× and 125× of these volumes were added to 1 ml of whole blood for comparison with the volumes when no additions were made. The determinations were made at the time periods of immediately after addition, 30, 60, 90 and 120 min later.

8) Changes of polyamine levels by cut off site of capillary
   (1) Polyamines in pellets of 6 fractions were measured after cutting the capillary tube at six 1 cm intervals from the bottom.
   (2) Characteristics of polyamine pattern in erythrocytes, lymphocytes and granulocytes: As it could be expected that

there would be an intermixing of leucocytes in fraction 6, leucocytes were treated as follows prior to study.

Step I Separation of lymphocytes[16]

After drawing blood into a heparinized tube, it is slowly layered upon Ficoll-Angioconray separating solution in a separate tube. This is subjected to centrifugation (1700×G, 3 min) and the lymphocyte layer sandwiched between the separating solution and plasma is collected using a capillary pipette.

Step II Separation of granulocytes

The upper portion of the erythrocyte layer is collected with a capillary pipette in accordance with the original method of Drew[12], which is then mixed together with PBS-NH_4Cl solution in a Fischer tube. After incubation for 20 min at 37°C, the remaining erythrocytes are hemolyzed and removed, after which the contents are separated by centrifugation at 1500×G for 30 sec. This procedure is repeated twice after which the granulocyte layer is collected. Samples for analysis are prepared from the leucocyte and granulocyte layers in accordance with the procedures used to prepare the erythrocyte sample.

(3) Changes in polyamine levels in anemic and hemorrhagic rabbits: As it was confirmed that the polyamine levels changed in accordance with specific gravity of erythrocytes, the following experiment was undertaken.

Preparation of anemic rabbits[19]: 1% Phenylhydrazine hydrochloride (PHZ) is injected subcutaneously into the femur in doses of 1 ml/kg/day for a period of 11 days. Venous blood is drawn from the ear into heparinized tubes prior to injection and 2, 3, 5, 9, 10 and 11 days after the series of injections. These are used as samples for determination of erythrocyte polyamines.

Preparation of hemorrhagic rabbits: About 50 ml of venous blood is drawn from the ear. Samples for determination of polyamines in erythrocytes are prepared by drawing venous blood from the ear into heparinized tubes prior to and 1, 2 and 3 days later.

RESULTS

1) Changes of free amino acids and polyamines in erythrocytes due to preservation, washing, addition of amino acids ect..

Total amino acid levels except Arginine (Arg) in plasma increased under room temperature and refrigerator preservation, while in erythrocytes Alanine (Ala), Valine(Val), Isoleucine(Ile), Leucine(Leu), Tyrosine(Tyr), Phenylalanine(Phe) increased, but the others decreased. However, the changes was large when kept in excess of 3 hr. The change was great-

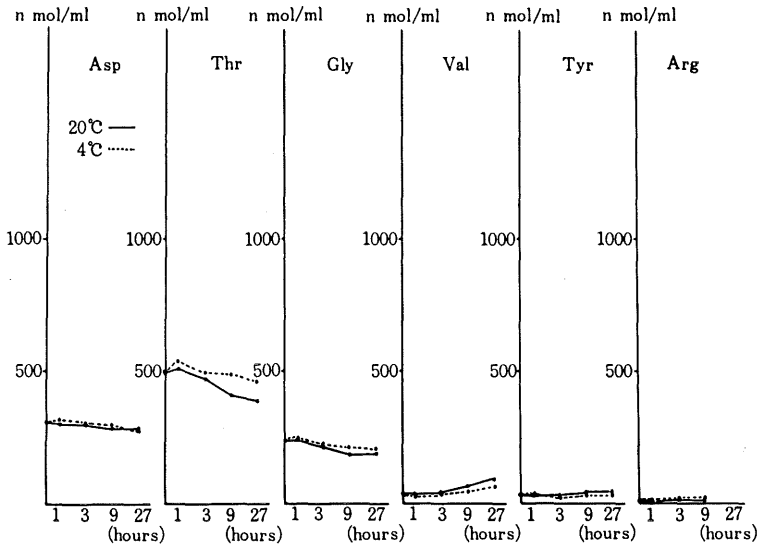


Fig. 3. Amino acids in erythrocytes
Study on length and temperature for preservation

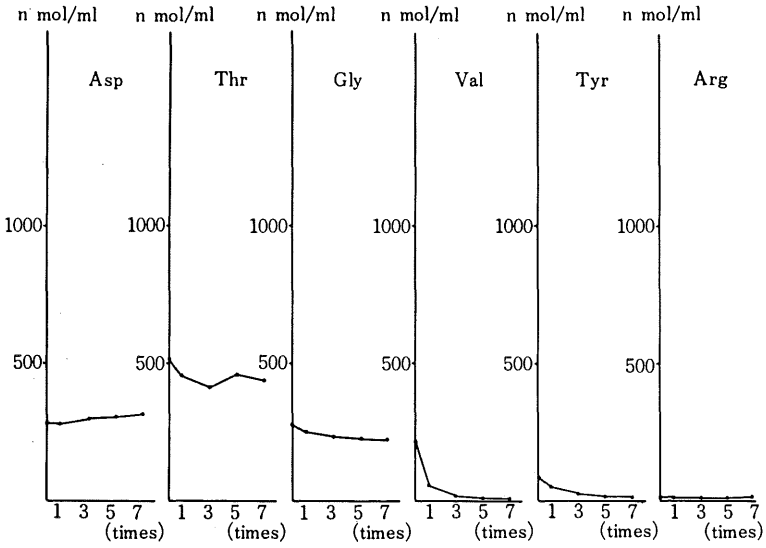


Fig. 4. Amino acids in erythrocytes
Changes with number of washings

er for room temperature preservation than for refrigerator storage. Fig. 3 shows Aspartic acid (Asp) as an ionic acidic amino acid with a side chain, Threonine (Thr) as a non-ion amino acid with a side chain which has an OH group, Glycine (Gly) as a non-ion amino acid without alkyl group, Val with an aliphatic side chain, Tyr with an aromatic side chain and Arg as a basic amino acid with an ionic side chain. Almost all amino acids decreased following washings, especially the branched chain amino acids decreased about 20% on one washing. Polyamine levels of Put, Spd and Spm decreased with washings, and the concentration decreased according to the number of washings (Figs. 4 and 5). The concentration of amino acids in erythrocytes showed increases or decreases by almost 10% following the addition of amino acids, but Val, Ile, Leu, Arg increased 2-5 fold (Fig. 6). The erythrocyte/plasma rate showed a drop in the total amino acid rate to below 50% as the addition increased from 30 to 90 and 180 μl (Fig. 7). When polyamines were added to whole blood, the Put value showed wide dispersion regardless of the volume added. However, as the polyamine volume was less than 200 p moles, this is considered within the range of measuring error. Spd and Spm in erythrocytes increased in proportion to the volume added, and it was considered this was due to exogenous effects (Fig. 8). However, according to reports of many investigators, Spd and Spm in erythrocytes were more than 10-fold voluminous than in plasma[44]. Actually speaking, however, it cannot be considered that the polyamine level in plasma was 125 times more voluminous than in erythrocytes. From the above experiments, it was considered that the erythrocytes should be treated as soon as possible after being drawn, and within 3 hr even when under refrigerator preservation. Also as in-

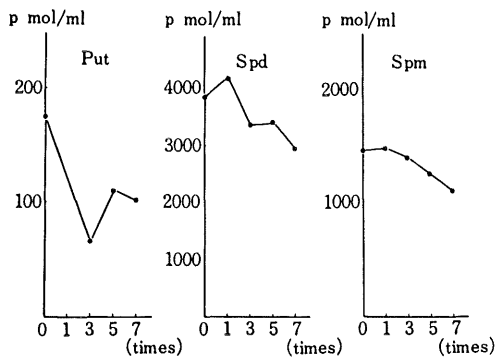


Fig. 5. Polyamines in erythrocytes
Changes with number of washings

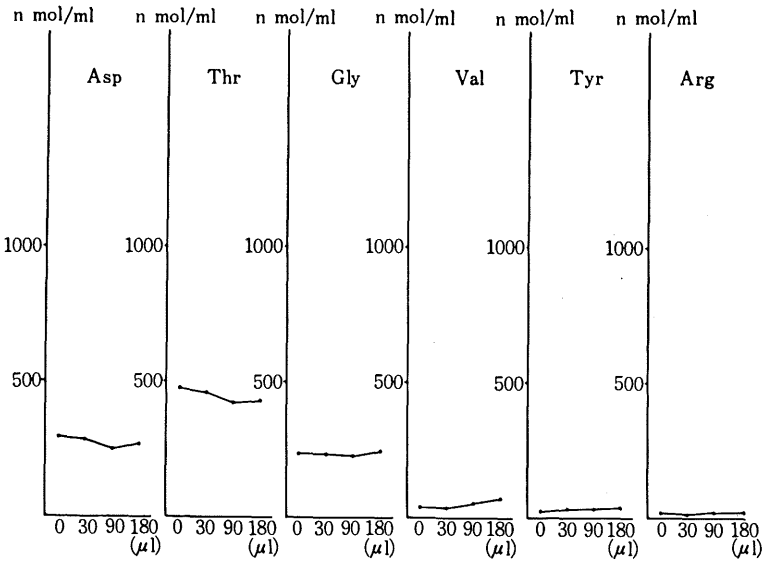


Fig. 6. Amino acids in erythrocytes
Amino acid compounds were added to whole blood

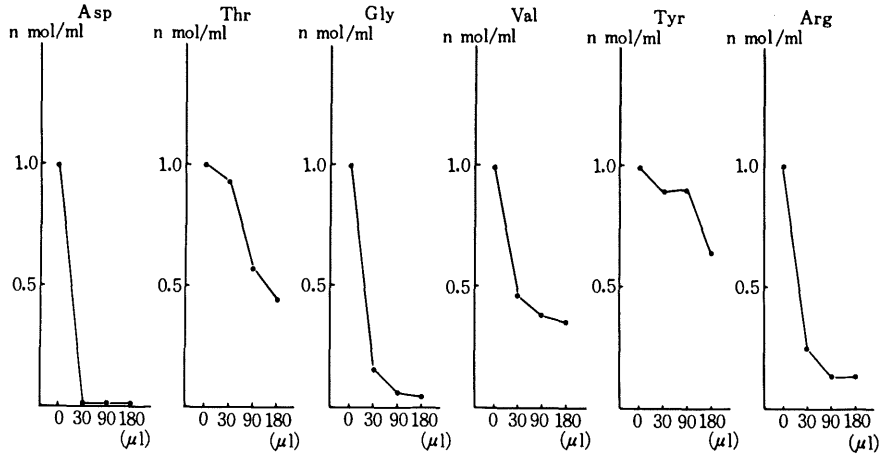


Fig. 7. Comparison of amino acids in erythrocytes and plasma
Amino acid preparation was added to whole blood
(Erythrocyte/plasma)

creases or decreases in concentration of branched chain amino acids in erythrocytes were observed following cell washing or adding of amino acids to whole blood, the loss of free amino acids, especially branched chain amino acids cannot be prevented by changing the washing procedure.

2) (1) Changes in polyamine values at cut surface of tube with normal erythrocytes (Fig. 9): The polyamine values in the 6 sections taken at lengths of 1 cm each from the bottom (high specific gravity fraction) upwards (low specfic gravity fraction) of the capillary tubes show increases as can be seen in the figure, indicating that the polyamine values vary by location within the tube. Spd values and Spm values in the upper section were 10 and 5 times greater respectively than those in the bottom. In view of the above results and the

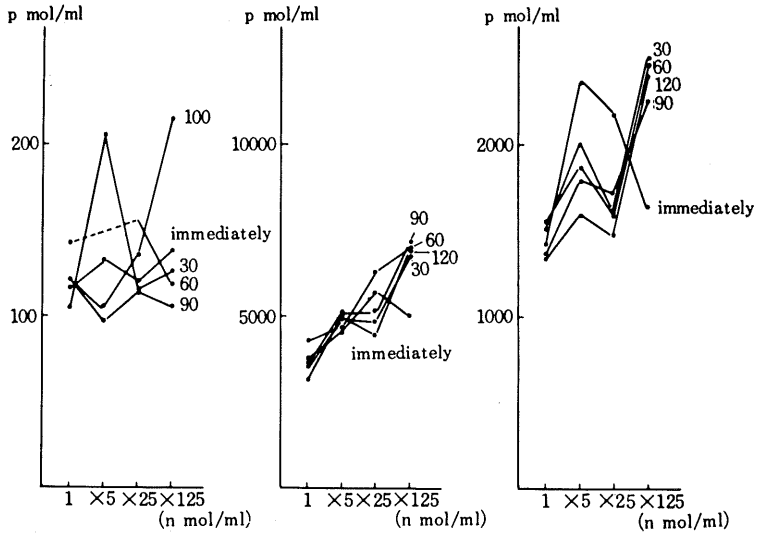


Fig. 8. Polyamines in erythrocytes
Polyamines were added to whole blood

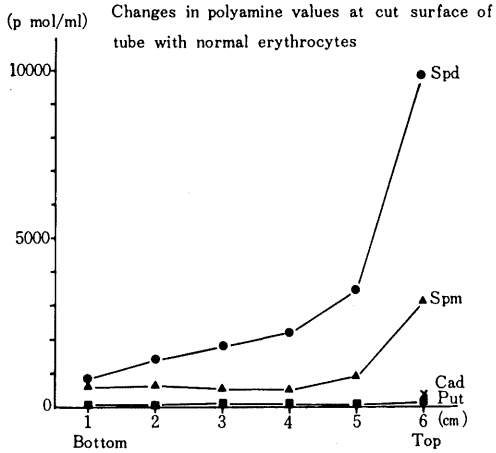


Fig. 9. Changes in polyamine values at cut surface of tube with normal erythrocytes
●—● Putrescine, ×—× Cadaverine,
●—● Spermidine, ●—● Spermine

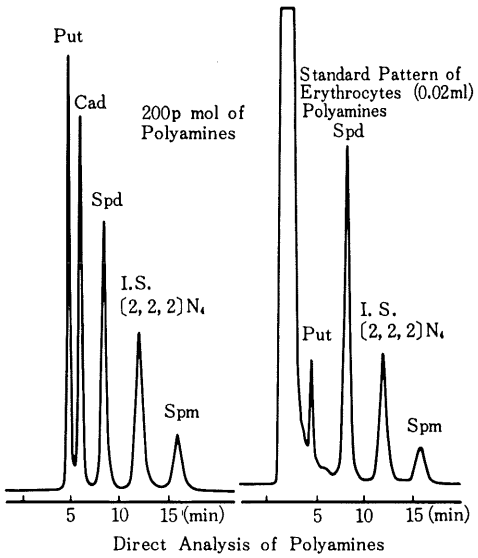


Fig. 10. Direct analysis of polyamines
Column: 4×80 mm of TSK, GEL, IEX 215
Elution Solvent: 0.35N Citrate Buffer pH5.28+2M NaCl+20% MeOH
Detection: OPA Method

fact that the changes in polyamine values are greater in the top than in the bottom section, we decided to carry out clinical examinations on fraction 6.

(2) Characteristics of the polyamine pattern in erythrocytes and leucocytes[51] (Figs. 10 and 11) The polyamine patterns in standard solution for polyamine determination and in erythrocytes are as shown in Fig. 12. As the Put value in standard solution was small, 100 p moles of Put was added to the solution. This standard solution was used to check the state of separation by HPLC. The ratio of Spd in erythrocytes was high (Cadaverine (Cad)/Spd 0, Spm/Spd 0.39), whie in granulocytes that of Spm was high (Spm/Spd 5.4) and in lymphocytes that of Cad was high (Cad/

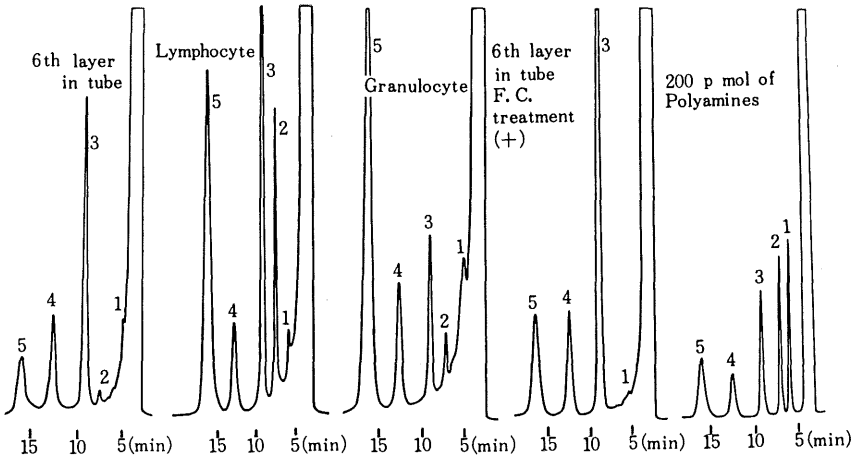


Fig. 11. Polyamine analysis in fraction 6
polyamine standard solution, fraction 6 layer (treated with Ficoll-Conray), granulocyte, lymphocyte, fraction 6 layer (not treated with Ficoll-Conray) from right to left, Compound: 1 Putrescine, 2 Cadaverine, 3 Spermidine, 4 [2·2·2]N_4·4HCl, 5 Spermine

Spd 10.5). These findings appear to indicate that in addition to erythrocytes, there is a mixture of granulocytes and lymphocytes in fraction 6, which are superimposed in the polyamine pattern. Photo. 3 shows supravital staining of fraction 6, and reticulocytes, lymphocytes and granulocytes can be observed.

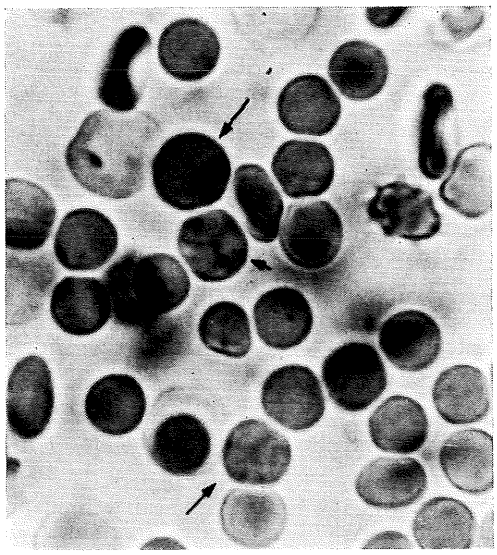


Photo. 3. Reticulocytes

(3) Changes in polyamine value in anemic and hemorrhagic rabbits (Table 3):

Anemic rabbits: At 1 week after 1% PHZ injection, reticulocytes could be observed microscopically in erythrocytes in more than 90% using methylene blue stain and Wright Giemsa stain. Further, there was a definite increase in polyamine values, particularly Spd, after injection. From the 3rd day after injection, the Spd value in fractions 1 and 6 began to increase, which indicates that reticulocytes began to constitute the greater majority of erythrocytes within these tubes.

Hemorrhagic rabbits: Spd and Spm values increased as reticulocytes increased in peripheral blood after bleeding. On the basis of the above experiments, mature erythrocytes collected at the bottom and immature erythrocytes gathered at the top of the capillary tube, and the polyamine value became higher in the younger cells.

(4) Free amino acid and polyamine values in erythrocytes (Fig. 12):

Free amino acid values: [1] The volumes of Asp and Methionine sulfoxide (oxidised Methionine: Met-SO) were greater in erythrocytes than in plasma. [2] The volumes of Val, Methionine (Met) Ile, Phe, Tryptophan (Trp) and Arg were less than found in plasma being only about 1/3. In general, there

Table 3. Changes in polyamine value in anemic rabbit due to PHZ (left) and hemorrhagic rabbit (right)
(unit: 10^2 p mol/ml)

		Anemia due to drug											Bleeding			
fraction 9	Cad	84	230	30	19				16	46	9		22	12	64	89
	Spd	107	358	389	504				755	795	784		88	120	173	330
	Spm	14	96	37	43				51	49	91		7	13	35	69
fraction 1	Cad	6	1	2	1				1	1	3		1	1	1	2
	Spd	75	17	211	534				830	969	750		98	109	96	104
	Spm	8	13	8	16				24	18	38		6	8	4	6
Erythrocyte ($\times 10^4$/mm)			314						190	176	130		660	470	395	395
1% PHZ (ml/kg/day)			+	+	+	+	+	+	+	+	+	+	Bleeding 50 ml			
Day		(−)	1	2	3	4	5	6	7	8	9	10	11 (death) 12		1	2	3

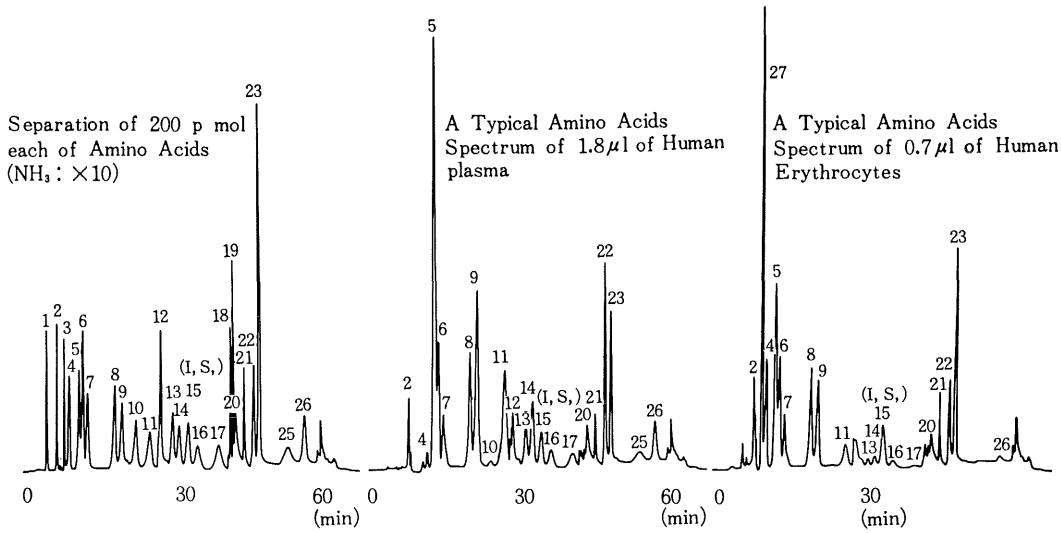


Fig. 12. Free amino acid pattern in plasma and erythrocytes
left: standard solution, middle: plasma
right: erythrocytes
Compounds: 1 CySO3H, 2 Tau, 3 S, CM. Cys, 4 Asp, 5 Thr, 6 Ser, 7 Glu, 8 Gly, 9 Ala, 10 n-BA, 11 Val, 12 Met, 13 Ile, 14 Leu, 15 Nle (I. S.), 16 Tyr, 17 Phe, 18 AIBA, 19 GABA, 20 His, 21 Orn, 22 Lys, 23H3, 24 E. A., 25 Trp, 26 Arg, 27 Met-SO

was an abundance of acidic amino acids, while there tended to be only a small volume of branched chain amino acids (Table 4).

Polyamine values: Spd and Spm values in fraction 6 were 10 and 5 times greater respectively than those in fraction 1. Put and Cad were also present in fraction 6 (Table 5).

DISCUSSION

In 1974, M. Sasaki et al.[39] developed the capillary sampling system (C-S system) for analysis of substances in erythrocytes. This method provides for analysis of information not heretofore available on intracellular (erythrocytes) substances. This information had been disregarded at the time of clinical examination and treatment in the past. Another characteristic of this method is that it is simple to perform. Comparison with conventional methods was made on cell treatment, and it was found to differ in the following respects.

(1) Exclusive centrifuge: The propylene tubes

Tab$_a$e 4. Range of normal amino acid levels in human plasma & erythrocytes

n=30, n mol/ml

	Plasma Mean ±2SD	CV (%)	Erythrocyte Mean ±2SD	CV (%)	E/P
Taurine	61± 30	49	56± 32	57	0.9
Met-SO			992±580	58	
Aspartic acid	5± 4	80	184±176	96	36.8
Threonine+ Glutamine	628±140	22	443±214	48	0.7
Serine+ Asparagine	160± 60	38	180±106	59	1.1
Glutamic acid	60± 28	46	203±152	75	3.4
Glycine	191± 70	37	301±138	46	1.6
Alanine	340±174	51	270±118	44	0.8
Valine	221± 80	36	32± 32	100	0.1
Methionine	22± 10	45	8± 8	100	0.4
Isoleucine	68± 26	38	9± 9	100	0.1
Leucine	116± 44	38	16± 16	100	0.1
Tyrosine	59± 28	47	32± 24	75	0.5
Phenylalanine	54± 18	33	12± 8	67	0.2
Histidine	72± 18	25	51± 51	100	0.7
Ornithine	43± 22	51	101± 72	72	2.3
Lysine	151± 40	26	117± 90	77	0.8
NH_3	128±128	100	340±314	92	2.7
Tryptophan	94± 38	40	14± 8	57	0.1
Arginine	78± 36	46	17± 12	71	0.2
Total	2555		3378		1.3

Table 5. Range of normal polyamine levels in human erythrocytes

(Mean±2SD, n=17, p mol/ml)

	Putrescine	Cadaverine	Spermidine	Spermine
1	—	—	512.6± 488.6	285.8± 284.6
2	—	—	756.5± 691.4	350.6± 296.6
3	—	—	1073.3± 1073.3	379.5+ 292.4
4	—	—	1557.4± 1525.2	492.6± 421.4
5	—	—	2863.2± 2863.2	760.4± 554.2
6	217.6± 217.6	309.6 ± 309.6	5592.7± 5000.6	1729.8± 1665.2

became heated following high speed centrifugation, thus causing the tube to expand, resulting in loss of uniformity in blood volume. Sasaki et al. installed an exclusive rotor into a Sorvall refrigerating centrifuge and succeeded in maintaining the temperature at about 4°C within the rotor. We confirmed that conventional high speed refrigerating centrifuge could be used if the temperature was dropped to about 4°C in advance. Therefore, we widened the grooves in a rotor for hematocrit measurement to 3 mm for this purpose.

(2) Exclusive capillary cutter: This is to cut propylene tubes into predetermined lengths. As it was difficult to cut both ends of the tube perpendicular, it was placed upon a vinylchloride plate as shown in Photo 2.

(3) For determination of free amino acids in erythrocytes, the cut tubes were attached to a dispensor, and the erythrocyte pellet was flushed out into a spitz test tube with 10 ml of phosphate saline. After shake-mixing, the samples were centrifuged (1600×G, 3 min, 20°C). the supernatant was aspirated with a pipette and cast away. This operation was repeated three times.

The difference between (1) and (2) was due to the apparatus and this problem has been resolved, but the difference in (3) still poses a problem, even when allowance was made for the difference between the determinants, i. e. amino acids, electrolytes and enzymes. This was due to free amino acid transport by erythrocyte membrane. Hagenfeldt et al.[18] (1980) contended that there existed three amino acid transport systems for erythrocytes, i. e., I) anion amino acids (Aspartate, Glutamate), in which Erythrocyte/Plasma (E/P) ratio was high. II) A system involving Ser, Gly and Ala migration in which, E/P ratio was less than 2. A positive correlation existed between plasma and erythrocytes even after washing. III) In the L system consisting of Met, Phe, Lys, Glu, the erythrocyte concentration was unrelated to plasma concentration, and could be readily flushed out by washing. By washing, in our experiments, all amino acids decreased except Asp and Arg, which increased slightly, but were within the range of measurement error. The decreases in Val, Ile, Leu were marked. Therefore, as the method of free amino acid determination in erythrocytes, we concluded that it would be possible to eliminate the effect of washings, if after high speed refrigerating centrifugation, the space rate of erythrocytes which were packed in stone-wall like fashion in propylene tubes was calculated (2. 4% according to Sasaki et al.), and

then, free amino acids in plasma subjected to washing were subtracted from free amino acids not washed.

Next, with reference to the free amino acid pattern in erythrocytes, Yoshino et al.[57] (1977) reported that high concentration of Asp, Ala, Glutamine (Gln), Gly, Glutamic acid (Glu) etc. were determined by amino acid analyzer after washing and treatment. Levy et al.[25] (1971) reported that Asp and Glutathionine were present in large quantities in erythrocytes, but were under detectable limits in plasma. Glu, Serine (Ser) and Thr were also found in large quantities in erythrocytes, but contrarily, Cystine, Met and Arg were present in smaller volumes than in plasma. He stressed the reason for the high acidic amino acid values was intracelluar trapping, and that determination in erythrocytes was accelerated in Asparagine Asn) and Gln and as a result were converted into Asp and Glu. Our method of determination also demonstrated the presence of a lagre volume of Met-SO and Asp in erythrocytes. Furthe, Bergström[3] (1974) measured the concentration of free amino acids in human muscle tissue and noted that Taurine (Tau), Glu and Gln were especially high, while Val, Leu, Phe, Citrulline and Tyr were low, being less than 2, while that of the remaining amino acids ranged from 5 to 10 in intracellular/extracellular concentration (IC/EC) ratio. Also the essential amino acids in muscle amino acid pool were a mere 8.4% with Gln, Glu and Ala comprising about 79%. It was thus considered that the amino acid pattern in muscle tissue resembled that in erythrocytes. Changes in polyamines due to aging of erythrocytes were confirmed by applying the capillary sampling system and measuring polyamines in erythrocyte using HPLC. Cooper et al.[10] (1976) also reported on separation of erythrocytes by centrifugation of blood drawn and packed into polypropylene tubes, 13×110 mm, which were spun for one hr at 39000×G, 30°C in an SS 34 angle head rotor. The desired fraction was carefully aspirated from the supernatant with a syringe. This was divided into top layer 10%, 4 layers of 20% each, and the bottom 10% layer. These were deproteinized and determined by Durrum 500 amino acid analyzer. The results showed that erythrocytes could be separated into age groupings by centrifugation with reticulocyte collecting at the top of the tube and aged erythrocytes accumlating at the bottom. Besis et al.[4] (1973) reported that aging of erythrocytes brings about decrease of membrane lipid and intracellular K/Na ratio, increase of methemoglobin, decrease of enzyme activity in glycolysis and decrease in spherocytosis and cell volume.

Thus, it is presumed that not only polyamines, but also the various erythrocyte components change with aging.

Parts of this study were presented at 19th Symposium of Clinical Chemistry (September 1979, Osaka, Japan) and at the 17th General Meeting of the Japanese Clinical Metabolism Society (January 1980, Osaka, Japan)

ACKNOWLEDGEMENT

In closing, I would like to express my profound appreciations to Professor Haruo Ezaki, Second Department of Surgery, Hiroshima University School of Medicine and to Professor Terumi Nakajima, formerly Institute of Pharmaceutical Sciences, Hiroshima University School of Medicine and presently Division of Molecular Biology, Institute of Medical and Dental Engineering, Tokyo Medical and Dental University for their continued kind guidance and review of this manuscript. I am grateful also to Associate Professor Hisanobu Yoshida, Institute of Pharmaceutical Sciences, Hiroshima University School of Medicine for his direct guidance.

REFERENCES

1. **Akimoto, M., Tokunaga, A., Ushio, H., Toishikawa, K., Takeichi, N., Sasaki, N., Matsuyama, T., Ezaki, H., Ito, H., Yoshida, H. and Nakajima, T.** 1980. Polyamine analysis of thyroid gland. J. Hiroshima Med. Ass. **33** : 1099-1104.
2. **Akimoto, M., Tokunaga, A., Hiraoka, T., Ushio, H., Toishikawa, K., Takeichi, N., Sasaki, N., Matsuyama, T., Ezaki, H., Ito, H., Yoshida, H. and Nakajima, T.** 1981. Polyamine patterns of the thyroid disease. J. Hiroshima Med. Ass. **34** : 149-151.
3. **Bergström, J., Furst, P., Noree, L-O. and Vinnars, E.** 1974. Intracellular free amino acid concentration in human muscle tissue. J. Appl. Physiol. **36** : 693-697.
4. **Besis, M. (translated by Weed R.I.)** 1973. Aging and death of erythrocytes, in living blood cells and their ultrastructure, p. 97-98. Springer Berlin.
5. **Biochemistry of diseases Editorial Committe**

Biochemistry of diseases. 1969 p. 1-71. *In* Nagai, K. (ed.), Erythrocyte Nakayama Shoten Co. Ltd., Tokyo.

6. **Chun, F. W., Rennert, O. M., Saffen, E. E. and Jaylor, W. J.** 1976. Effect of polyamines on the electrokinetic properties of red blood cells. Bioche. Biophys. Res. Commu. **69** : 1095-1101.
7. **Cohen, S. S.** 1971. Introduction to the polyamines Prentice-Hall, Inc. Englewood Cliffs New Jersey.
8. **Cohen, L. F., Lundgren, D. W. and Farrell, P. M.** 1976. Distribution of spermidine and spermine in blood from cystic fibrosis patients and control subjects. Blood **48** : 469-475.
9. **Cooper, K., Shukla, J. B. and Rennert, O. M.** 1978. Polyamine compartmentalization in various human disease states. Clin. Chem. Acta **82** : 1-7.
10. **Cooper, K. D., Shukla, J. B. and Rennert, O. M.** 1976. Polyamine distribution in cellular compartments of blood and in aging erythrocytes. Clin. Chem. Acta **73** : 71-88.
11. **Dunzendorfer, U. and Russell, D. H.** 1978. Altered polyamine profiles in prostatic hyperplasia and in kidney tumors. Cancer Res. **38** : 2321-2324.
12. **Drew, S. I., Carter, B. M., Guidera, D. P. and Terasaki, P. I.** 1977. Autoimmune cytotoxic granulocyte antibodies in health and disease. Transplant. Proc. **9** : 1887-1894.
13. **Durie, B. G. M., Salmon, S. E. and Russell, D. H.** 1977. Polyamines as markers of response and disease activity in cancer chemotherapy. Cancer Res. **37** : 214-221.
14. **Endo, T., Shoji, K., Mitamura, K., Suzuki, H., Oda, T., Shimazu, H., Akaike, Y. and Samejima, K.** 1976. Determination of polyamines in blood and its application for a possible cancer diagnosis. Igakunoayumi **98** : 485-488.
15. **Fischer J. E.** 1976. Total Parenteral Nutrition. Little, Brown and Company Boston.
16. **Fukuda, Y., Dohi, K., Asahara, T., Ono, E., Nishimura, S., Takasugi, S., Fukuda, M. and Ezaki, H.** 1976. Methods of human histocompatibility testing, especially lymphocyte cytotoxicity test. J. Hiroshima Med. Ass. **29** : 851-859.
17. **Gaugas, J. M.** 1980. Polyamines in biological research. John Wiley Sons Ltd, Chichester New York Brisbane Toronto.
18. **Hagenfeldt, L. and Arridsson, A.** 1980. The distribution of amino acids between plasma and erythrocytes. Clin. Chem. Acta **100** : 133-141.
19. **Hara, H. and Ogawa, M.** 1976. Erythropoietic precursors in mice with phenylhydrazine-induced anemia. Am. J. Hemat. **1** : 453-458.
20. **Hara, S. and Tsuji, A.** 1978. Recent liquid chromatography -Basic knowledge and application- p. 119-135 Nanzando Co Ltd. Tokyo.
21. **Imamura, Y., Nomura, T., Yamaguchi, S. and Tsukada, R.** 1978. Diseases of the blood and the hematopoietic organs. p. 89-164 Chugai Igaku Co., Tokyo.
22. **Inoue, H.** 1972. Biosynthesis of polyamines and its regulation. Metabolism and Disease **9** : 1010-1017.
23. **Kanebuti, M.** 1967. Changes of protein metabolism in early stage after gastrectomy (anabolic function in catabolism). J. Jpn. Surg. Soc. **68** : 1831-1846.
24. **Kuriyama, K.** 1965. Changes of free amino acids in serum and urine before and after operation. J. Jpn. Surg. Soc. **66** : 1887-1914.
25. **Levy, H. L. and Barkin, E.** 1971. Comparison of amino acid concentrations between plasma and erythrocytes Studies in normal human subjects and those with metabolic disorders. J. Lab. Clin. Med. **78** : 517-523.
26. **Marton, L. J., Heby, O., Levin, V. A., Lubich, W. P., Grafts, D. C. and Wilson, C. B.** 1976. The relationship of polyamines in cerebrospinal fluid to the presence of central nervous system tumors. Cancer Res. **36** : 973-977.
27. **Maruta, Y.** 1970. Effect of a surgical operation and a shock on the levels of individual amino acids in serum. J. Jpn. Surg. Soc. **71** : 702-725.
28. **Matsuzaki, S. and Suzuki, M.** 1975. Thyroid function and polyamines 1, Thyrotropin stimulation of polyamine biosynthesis in the rat thyroid. Endocrinol. Jpn. **22** : 339-345.
29. **Matsuzaki, S., Suzuki, M., Hamana, Y. and Ito K.** 1979. Elevation of histamine and polyamine in cancer of thyroid gland. Clin. Endocrin. (Jpn.) **27** : 265-268.
30. **Miwa, K.** 1965. Changes of free amino acids in serum, especially Alanine, Leucine, Isoleucine and Valin fraction. J. Jpn. Surg. Soc. **66** : 1638-1663.
31. **Nishioka, K., Romsdahl, M. M. and McMurtrey, M. J.** 1977. Serum polyamine alterations in surgical patients with colorectal carcinoma. J. Surg. Oncology **9** : 555-562.
32. **Nita, M., Nakanishi, Y., Higami, M., Mitomo, Y. and Yamamoto, M.** 1979. Polyamine and aging. Igakunoayumi **109** : 552-554.
33. **Perin, A. and Sessa, A.** 1978. Changes in polyamine levels and protein synthesis rate during rat liver carcinogenesis induced by 4-dimethylaminoazobenzene. Cancer Res. **38** : 1-5.
34. **Russell D. H. and Durie B. G. M.** 1978. Polyamines as biochemical markers of normal and malignant growth. Raven Press, New York.
35. **Russell D. H.** 1971. Increased polyamines concentrations in the urine of human cancer patients. Nature new biology **233** : 144.
36. **Sabiston D. C.** 1972. Textbook of Surgery the biological basis of modern surgical practice. W. B. Saunders Company, Philadelphia, London, Toronto
37. **Saeki, Y., Uehara, N. and Shirakawa, S.** 1978.

Sensitive fluorometric method for the determination of putrescine, spermidine and spermine by high performance liquid chromatography and its application to human blood. J. Chromatogr. Biomed. Appl. 145 : 221-229.

38. **Sasaki, M.** 1980. Pathological changes and simple determination of the chemical components in erythrocytes -development of capillary sampling system-. Recent clinical chemistry Gakkai Shuppan Center, Tokyo.
39. **Sasaki M.** 1974. Development of capillary sampling system for the determination of the chemical components in erythrocytes. Jap. J. Clin. Path. 22 (Suppl.) 197.
40. **Satoh, H., Ogoshi, S. and Usui, S.** 1977. Atlas of total parenteral nutrition with simple technique. Igakushoin Ltd., Tokyo.
41. **Savory, J., Shipe, J. R. and Wills M. R.** 1979. Polyamines in blood -cells as cancer markers. The Lancet 24 : 1136-1137.
42. **Schwartz, S. I.** 1974. Principles of Surgery. McGraw Hill Book Company.
43. **Sekiya, T.** 1969. Studies of protein metabolism following surgical injuries, administration of glucocorticoid and fasting. J. Jpn. Surg. Soc. 70 : 380-404.
44. **Shipe, J. R. and Savory, J.** 1980. High performance liquid chromatographic separation and fluorescence detection of polyamines in plasma and erythrocytes. Ann. Clin. Lab. Sci. 10 : 128-136.
45. **Swendseid, M. E., Panaqua, M. and Kopple, J. D.** 1980. Polyamine concentrations in red cells and urine of patients with chronic renal failure. Life Sci. 26 : 533-539.
46. **Takami, H., Kodaira, S., Ishibiki, H. and Abe, R.** 1981. Relationship between prognosis of patients after curative reaction of colon cancer and preoperative polyamine and CEA levels in serum. J. Clin. Surg. (Jpn.) 36 : 833-837.
47. **Takami, H., Romsdahl, M. and Nishioka, K.** 1979. Polyamines in blood-cells as a cancer marker. The Lancet 27 p. 912.
48. **Takeda, Y., Matsuzaki, K., Hosokawa, K., Miwatani, T., Tominaga, K., Kitamura, M., Taguchi, T. and Shiba, S.** 1974. Possible diagnosis of cancers by determination of urinary polyamines. Igakunoayumi 88 : 391-394.
49. **Terabayashi, T.** 1976. Blood levels of polyamines in patients with brain tumor: special reference to relationship between blood levels of polyamines and histological findings, extension as well as treatment of tumor. Neurol. Med. Chir. 16 : 43-50.
50. **Tokunaga, A., Akimoto, M., Matsuyama, T., Kodama, M., Ezaki, H., Yoshida, H. and Nakajima, T.** 1981. Microdetermination of amino acids and polyamines in human erythrocytes (1) Establishment of a simple and rapid analysis. J. Hiroshima Med. Ass. 34: 239-244.
51. **Tokunaga, A., Akimoto, M., Ono, E., Matsuyama, T., Kodama, M., Ezaki, H., Yoshida, H. and Nakajima, T.** 1981. Determination of amino acids and polyamines in human erythrocytes Differentiation of polyamines in erythrocytes sediment in a capillary tube (especially concerned to the aging of the cells), Igakunoayumi 118 : 925-928.
52. **Uehara, N., Shirakawa, S., Uchino, H. and Saeki, Y.** 1980. Elevated contents of spermine and spermidine in the erythrocyte of cancer patients. Cancer 45 : 108-111.
53. **Yonezawa, Y., Hiramatsu, Y., Hayase, R., Ogawa, T., Eguchi, K. and Sekiba, K.** 1981. Study on polyamine metabolism in embryogrowth. World Obstet. Gynecol. 33 : 912-914.
54. **Yoshida, H., Nakajima, T., Ueno, Y., Koine, N., Onda, M., Ohe, K. and Miyoshi, A.** 1978. A simple and rapid screening method of amino acids and amines in biological samples. Hiroshima J. Med. Sci. 27 : 85-92.
55. **Yoshida, H. and Nakajima, T.** 1978. Homemade apparatus for high performancy liquid chromatography. Jap. Biochem. Soc. 50 : 588-594.
56. **Yoshida, H. and Nakajima, T.** 1980. High performancy liquid chromatography analysis of amino acids and amines and its application for the clinical chemistry. Recent Advances in clinical chemistry p. 323-338. Gakkai Shuppan Center, Tokyo.
57. **Yoshino, K. and Koike, H.** 1977. Free amino acids in plasma and erythrocytes Values of 20 diseased controls as compared with normal control data in the literature. J. Kyorin Med. Soc. 8 : 227-231.