

FIRST APPARENT DISSOCIATION CONSTANT OF CARBONIC ACID,
 pK_1' , IN HUMAN SERUM AND RED CELLS

by

Arthur A. Messier

NAVAL SUBMARINE MEDICAL RESEARCH LABORATORY
REPORT NUMBER 780

Bureau of Medicine and Surgery, Navy Department
Research Work Unit MR041.01.01-0125B9XX.03

Transmitted by:

Karl E. Schaefer

Karl E. Schaefer, M. D.
Head, Environmental Physiology Branch

Reviewed and Approved by:

Charles F. Gell

Charles F. Gell, M.D., D.Sc. (Med)
SCIENTIFIC DIRECTOR
NavSubMedRschLab

Approved and Released by:

R. L. Sphar

R. L. Sphar, CDR MC USN
OFFICER IN CHARGE
NavSubMedRschLab

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SUMMARY PAGE

PROBLEM

To determine the first apparent dissociation constant of carbonic acid (pK_c') for human erythrocytes. Using the derived pK_c' , develop a simple method for the determination of erythrocyte bicarbonate concentration.

FINDINGS

pK_c' was determined in venous blood of ten healthy, adult, male human subjects. Prior to the determination of pK_c' , the blood sample was divided and equilibrated into the following portions, 1) As drawn from the antecubital vein, 2) Oxygenated - with a 5% CO_2 , O_2 balance gas mixture, and 3) Reduced - with a 5% CO_2 , N_2 balance gas mixture. The pK_c' of the As drawn, Oxygenated, and Reduced samples were found to be 6.144, 6.187, and 6.157, respectively.

Using these pK_c' values along with the directly determined values of pH and P_{CO_2} , it was then possible to derive a simplified method of determining the erythrocyte bicarbonate concentration.

APPLICATION

There have been very few studies of pK_c' in the literature. This report will be of value to those interested in clinical and research applications. The method outlined for the determination of cell bicarbonate may be applied to correlated studies of serum or red cell electrolyte changes extant during FBM submarine patrols or hyperbaric exposure.

ADMINISTRATIVE INFORMATION

This investigation was conducted as part of Bureau of Medicine and Surgery Research Work Unit MR041.01.01-0125B9XX. The present report is Number 3 on this work unit. It was submitted for review on 26 February 1974, approved for publication on 20 March 1974 and designated as NavSubMedRschLab Report No. 780.

This report constitutes a copy of the thesis of Mr. Arthur A. Messier, submitted to, and accepted by the Division of Biomedical Sciences at Brown University, in partial fulfillment of the requirements for the degree of Master of Sciences in Cell Physiology and Biophysics.

All of the research work was done in the Naval Submarine Medical Research Laboratory.

ACKNOWLEDGEMENT

The author wishes to express profound thanks and appreciation to Dr. Joseph D. Bloom and to Dr. Charles F. Gell for their continued interest and support and to Dr. Karl E. Schaefer, thesis advisor, for guidance needed to complete this study.

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ABSTRACT

The first apparent dissociation constant of carbonic acid, pK_1' of serum and red cells was determined on venous blood of ten healthy, adult, male, human subjects. The pH and P_{CO_2} of serum and red cells were analyzed electrometrically and a micromanometric method was used for the determination of total carbon dioxide content. Erythrocyte carbamino hemoglobin levels were estimated and used for the correction of erythrocyte pK_1' . Each blood sample was subjected to the following regimen before centrifugation, 1) As Drawn from the antecubital vein, 2) Oxygenated with a 5% CO_2 , O_2 balance gas mixture, and 3) Reduced with a 5% CO_2 , N_2 balance gas mixture.

pK_1' of serum and red cells are presented:

		<u>AS DRAWN</u>	<u>OXYGENATED</u>	<u>REDUCED</u>
SERUM (N=10)	Mean	6.099	6.125	6.105
	SD	.028	.026	.026
RBC (N=10)	Mean	6.144	6.187	6.157
	SD	.039	.040	.032

The consistently larger values for red cell pK_1' than the respective serum data may be attributed to the greater amount of carbamino hemoglobin concentration present in the erythrocytes.

A simplified method for the calculation of a carbamate-corrected, erythrocyte bicarbonate concentration using the experimentally determined red cell pK_1' value has been formulated. The method involves the use of a regression equation relating serum and red cell pH, the equivalence of serum and red cell P_{CO_2} , along with the experimentally determined red cell pK_1' .

FIRST APPARENT DISSOCIATION CONSTANT OF CARBONIC ACID, pK_1' , in HUMAN SERUM AND RED CELLS

INTRODUCTION

The classical Henderson-Hasselbalch equation is used extensively in clinical and research applications for the assessment of respiratory and metabolic components of acid-base balance (Davenport, 1958). Knowledge of any two variables allows the calculation of the third variable in the equation. The first apparent dissociation constant of carbonic acid, pK_1' , is also involved in the derivation of standard bicarbonate by the Astrup equilibration technique (Astrup and coll., 1960).

The value for pK_1' in serum, pK_S' , has been extensively investigated and is accurately determined (Sinclair and coll., 1968).*

The pK_1' in red cells, pK_C' , is not necessarily the same as in serum. One reason may be that a considerable fraction of the CO_2 of red cells is combined with hemoglobin as carbamino hemoglobin, $HbCO_2$. The proportion of $HbCO_2$ depends upon the degree of oxygenation and on the hydrogen ion concentration (Dill, Daly, and Forbes, 1937). Recent evidence has also implicated the influence of 2,3 diphosphoglycerate, DPG, upon CO_2 affinity (Bauer, 1970, Siggaard-Anderson, 1971, Bauer and Schroeder, 1972).

Variations of pK_S' have also been related to alterations in ionic strength (Hastings and Sendroy, 1925), hemoglobin concentration (Margaria and Green, 1933), and oxygenation (Dill, Daly and Forbes, 1937). Hence pK_C' may conceivably be influenced by the same factors.

Early studies, Dill, Daly and Forbes, (1937), reported values of pK_C' which varied in reduced (5.98) and oxygenated (6.04) red cells and which differed significantly from the accepted value for pK_S' (6.11). Deane and Smith (1957) attempted to reconcile the discrepancies in the reported data by determining pK_1' in human red cells after correction for carbamino hemoglobin. These workers obtained a pK_C' of 6.18 on venous blood that was analyzed, as drawn, without prior gas tonometry.

Considering the limited number of studies and the variability of reported values for pK_1' of red cells, the value of pK_C' has been reinvestigated. pK_1' of serum has also been determined simultaneously in this study to provide a comparison with pK_C' and to assure the reliability of the technique.

METHODS

I. PROTOCOL

The serum and red cell carbonic acid pK_1' was estimated using the blood of ten healthy laboratory personnel

*The word "serum", in this paper, is used to describe the fluid separated by centrifugation from blood treated with heparin.

over a three-month period. All subjects were questioned as to the general state of their health and smoking history. Due to the extensive number of measurements that were made on each blood sample, one subject was run per day.

After a five to ten minute resting period twenty-five ml of venous blood was withdrawn, without stasis, from an antecubital vein. The heparinized, (1000 units/ml sodium heparinate), sample was then apportioned in the following manner (see Figure 1):

1) As drawn portion - this sample was analyzed as drawn from the body without further treatment. A two ml aliquot of whole blood from this sample was analyzed for:

Hematocrit (Hct)	pH
Hemoglobin (Hb)	P_{CO_2}
Oxyhemoglobin (HbO ₂)	P_{O_2}
Carboxyhemoglobin (HbCO)	% Saturation
O ₂ content (T_{O_2})	CO ₂ Content (T_{CO_2})
Chloride (Cl ⁻)	H ₂ O Content

The remaining sample was anaerobically transferred to completely fill a seven ml test tube containing 0.5 ml heavy mineral oil. The sample was then centrifuged at 37°C, 5500 rpm, for fifteen minutes. The resulting serum and red cells were then carefully separated and stored at 0-4°C before

being analyzed for: pH, P_{CO_2} , P_{O_2} , plasma Hb, T_{O_2} , T_{CO_2} , Cl⁻, and H₂O content.

2) Oxygenated portion - Eight ml of this sample was equilibrated with 5% CO₂ in O₂ for 40-45 minutes. After equilibration the blood sample was separated at 37°C. Analytical procedures were performed in the same manner as the as drawn serum and cell samples.

3) Deoxygenated portion - Eight ml of this sample was equilibrated with 5% CO₂ in N₂ for 40-45 minutes. The sample was then handled similarly as the oxygenated sample.

II. METHODOLOGY

A. Equilibrating Gases -

A gas mixing pump was used to prepare the desired equilibrating gases. After addition of the required gases, the gas cylinders were mixed on a gas cylinder rolling apparatus for a minimum of one hour. The gases were then analyzed in triplicate (SD ± 0.03 Vol %) using the Scholander gas analyzer (1948).

B. Tonometry -

A tonometer was used in a system similar as that described by Bartels and Harms (1959). An eight ml sample of blood was equilibrated for 40-45 minutes at 300 rev/min. The water bath was maintained at 37.25° ± 0.05 SD. The gas mixtures were either 5.51% CO₂ in O₂ or 5.72% CO₂ in N₂. The gas flow rate

FLOWCHART OF EXPERIMENTAL PROTOCOL

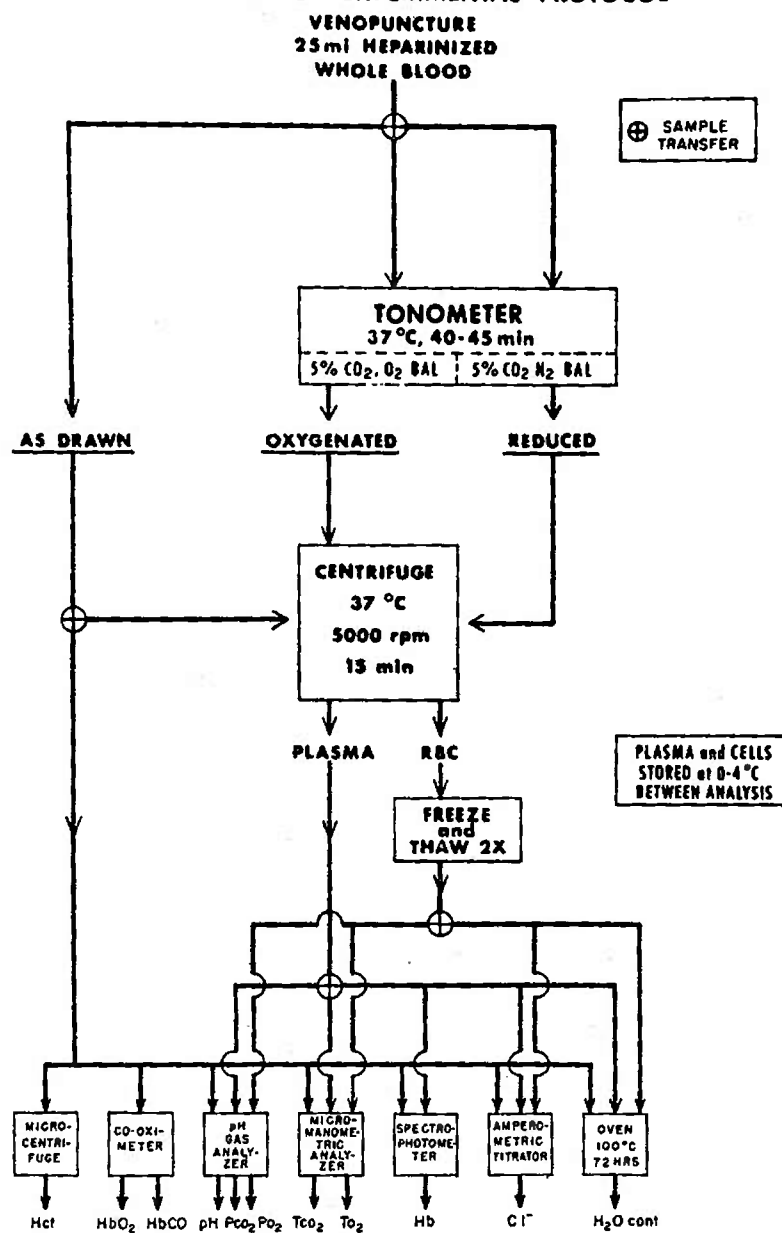


FIGURE 1

was 120-150 ml/min and the gases were saturated with water vapor before entering the tonometer. Sampling from the tonometer was done with a 10 ml syringe attached with a 17 gage 2 1/2 inch needle, the dead space of the syringe and needle was flushed and filled with 0.4 ml of the equilibrated sample prior to total extraction of the sample. Blood was then anaerobically transferred to a seven ml capacity test tube containing 0.5 ml heavy mineral oil, the tube completely filled, stoppered and centrifuged.

C. Centrifugation -

A refrigerated centrifuge* was modified to allow centrifugation at 37°C. The centrifuge was equipped with a heating coil and a thermostat which maintained the temperature of the centrifuge at 37° ± 0.5 SD.** The mean difference of pH and P_{CO_2} values between the equilibrated blood samples and the serum after centrifugation was $\Delta \pm 0.021$ pH and $\Delta \pm 1.2$ mm Hg respectively, indicating that no significant loss of CO_2 had occurred during the centrifugation.

D. Analytical Procedures -

Micromanometric analyses of total carbon dioxide content (T_{CO_2}) and total oxygen content (T_{O_2}) in both serum and red cells were determined with the apparatus described by Van

Slyke and Plazin (1961) on 50 μ l samples.

pH was measured using an Instrumentation Laboratory pH assembly (I.L. Model 113-S1). pH was calibrated with 3 ml ampoules of precision buffers (Radiometer, pH 6.839 ± .005, pH 7.383 ± .005), an additional precision buffer, pH 7.393 ± .005 (Accuval), was used to insure proper electrode response. For determination of red cell pH, the packed erythrocytes after being centrifuged were carefully transferred into a well greased glass syringe and were frozen and thawed 2-3 times. The pH of the lysate was measured with the same pH electrode as described above with the additional precaution of rinsing the pH sensitive glass with a 10% hypochlorite solution between each analysis to eliminate accumulation of protein deposition on the electrode. Recalibration of pH was performed before each reading. P_{CO_2} and P_{O_2} was measured directly using the I. L. Model 113-S1 blood gas analyzer. Calibration was accomplished using a minimum of three gases, recalibration was performed before each sample was analyzed. All calibration gases were analyzed within ± 0.03 volume per cent standard deviation. Prior to admitting a blood sample, a calibration gas with the approximate composition of the sample would be admitted; i.e., if a serum sample was equilibrated with 5% CO_2 in N_2 the same gas sample would be admitted to the chamber prior to the serum. The as drawn samples contained a 5% CO_2 , 12% O_2 , N_2 balance gas in the chamber prior to blood admission. The water content of each sample was determined by

* International High Speed IEC Model HR-1.

** Yellow Springs Instrument Co., Temperature Control Model 71, Tele-Thermometer Model 46TUC.

drying one ml of either serum or cells at 100°C for 72 hours. The chloride concentration of serum and cells was measured amperometrically using the Cotlove-Buchler chloride titrator. The hemoglobin concentration of whole blood was determined after conversion to cyanomethemoglobin and read at 540 Angstrom units with a Coleman Spectrometer Model 295. Serum hemoglobin levels were monitored to insure the integrity of the red cells. At no time did the hemoglobin concentration in the serum exceed 2% of the total concentration. The hematocrit was determined using microcapillary tubes and spinning at 3000 rev/min for 5 minutes using the Phillips-Drucker Model L411 microcentrifuge. Whole blood oxyhemoglobin (HbO₂) and carboxyhemoglobin (HbCO) saturation were determined with an Instrumentation Laboratory Model 182 CO-Oximeter.

Total amount of time taken for a complete run averaged six to seven hours. All samples were held at 0-4°C between analyses.

E. Calculations -

All calculations are based on a temperature of 37°C. All concentrations are reported as millimoles per liter of serum or red cells except for the Donnan ratios for chloride and bicarbonate which are derived in units of mEq/kg H₂O.

Serum and red cell pK₁' were calculated from the Henderson-Hasselbach equation.

$$(i) \text{pK}_1' = \text{pH} - \log \frac{(\text{T}\text{CO}_2 - \text{HbCO}_2 - \text{H}_2\text{CO}_3)}{\text{H}_2\text{CO}_3}$$

where pH is the negative logarithm of hydrogen ion concentration, TCO₂ is the total carbon dioxide content in mM/l. H₂CO₃ (α^PCO₂) is the carbonic acid concentration in mM/l which is obtained by multiplying the solubility coefficient of CO₂, α, by ^PCO₂ where α_s = 0.0307 (Austin, 1963) and α_c = 0.0260 (Bartels and Wribitzky, 1960); ^PCO₂ is the partial pressure of carbon dioxide, in mm Hg, measured electrometrically for either serum or erythrocytes.

The amount of carbamino hemoglobin (HbCO₂) in serum is at a measurable but minimal level, consequently correction for HbCO₂ in serum was not made. The total CO₂ content of the red cell was corrected for HbCO₂ at a ^PCO₂ of 40 mm Hg by assuming that 0.11 mM of CO₂ combines as HbCO₂ per mM of oxyhemoglobin and 0.33 mM of CO₂ as HbCO₂ per mM of reduced hemoglobin. An alternative method for the calculation of [HbCO₂] was also used and is outlined in Table 7 of the Results. The amount of oxygenated or reduced Hb, in grams, was calculated from the O₂ content and O₂ saturation by assuming that 1 gram of Hb combines with 1.39 ml of O₂.

pK₁' used in this equation and throughout this paper is the negative logarithm of the first apparent dissociation exponent of carbonic acid of either serum or cells obtained gasometrically. The pK₁' of this paper is the equivalent of the pK₁''' defined by Siggaard-Anderson (1962).

F. Statistics -

Comparison of the means of paired and unpaired data was analyzed, two-tailed, with Student's *t*-test. Differences were considered significant at $p < 0.05$.

In the preliminary analysis of the data, the two groups - 5 smokers and 5 non-smokers were compared. Since the only statistically significant difference between the two groups was their HbCO level, the combined grand mean of $N = 10$ is reported.

RESULTS

A flow chart of the experimental protocol is presented in Figure 1. Parameters that were calculated from the primary data (as seen in the last row of Figure 1) was then used to calculate the following parameters: H_2CO_3 , HCO_3^- , $HbCO_2$, $^{10}Cl^-$, $^{10}HCO_3^-$, and pK_1' .

Table 1 represents a summary of the physical data of the subjects. These subjects were all ostensibly normal, healthy males. A distinction was made regarding their smoking habits, five were classified as non-smokers and five as smokers. On the basis of the completed data analysis, the only statistically significant difference between these two groups was an expected difference in carboxyhemoglobin level. Consequently the two groups have been combined and the results expressed as a grand mean of ten normal subjects representing a population of smokers and non-smokers.

A random check of the accuracy and precision of the micromanometric technique was made on eight separate experimental days. A fresh 30 mM bicarbonate standard was prepared according to Van Slyke and Plazin's recommendation. The results of this test are shown in Table 2.

A preliminary experiment was performed in an attempt to determine the adequacy of using Vacutainers* for the determination of blood gases.

Blood was taken from an antecubital vein, without stasis, using a "butterfly" assembly**, and collected in an heparinized syringe. A 7 ml portion was transferred to a test tube containing 0.5 ml heavy mineral oil and capped. A second 7 ml was transferred to a 7 ml evacuated Vacutainer. The results of the blood gas analyses are depicted in Table 3. The sample transferred to a test tube containing mineral oil was not significantly altered from the control. The measured parameters of the sample transferred to the Vacutainer were all significantly different from the control. Consequently all samples which were equilibrated with either 5% CO_2-N_2 balance or 5% CO_2-O_2 balance were transferred into test tubes containing 0.5 ml heavy mineral oil, filled completely and stoppered. The mean differences for P_{CO_2} and P_{O_2} values of samples tonometered with 5% CO_2-N_2 balance were shown to be

* Registered Trademark, Becton-Dickinson and Co.

** Abbott Laboratories, Intravenous Injection Set

Table 1. Physical data and smoking history of subjects

Subject	Age yrs.	Weight lbs.	Height in.	Smoking History
1	29	172	70	Non-smoker
2	22	180	72	Non-smoker
3	25	160	68	Non-smoker
4	46	150	67	Non-smoker
5	28	170	70	Non-smoker
6	28	170	70	Pipe or cigar, occasional
7	31	171	72	Cigarettes, 1 pack/day
8	25	185	72	Cigarettes, 2 packs/day
9	26	170	68	Cigarettes, 1.5 packs/day
10	44	190	71	Cigarettes, 2 packs/day
Ave.	30.4	172	70	

± 1.5 mm Hg and ± 1.0 mm Hg respectively. Similar results for the mean difference of P_{CO_2} were found for the oxygenated samples. However, the mean difference for P_{O_2} was ± 50.5 mm Hg, which was due in part for the presence of a greater partial pressure of O_2 in the tonometer ($P_{O_2} = 673$ mm Hg) and subsequent loss in transfer from the tonometer to the sampling syringe.

Whole blood data is summarized in Table 4. Note that the carboxyhemoglobin (HbCO) level is the only parameter

which is significantly different, all other data have been combined after a prior statistical analysis showed no differences between smokers and non-smokers.

The data which have been utilized in the calculation of $pK_{s,c}$ for the AD, O, and R samples is outlined in Table 5. Note the larger standard deviation of the as drawn sample as compared to that of the tonometered samples. The SD for HCO_3^- also is much larger than the standard $NaHCO_3$ solution (see Table 2).

Table 2. Analyses of 30mM standard NaHCO_3 samples measured from 50 μl mark of Stopcock Pipet. Standards were analyzed on same day as pK_1' Determinations

Date	P _{CO} ₂ from duplicate p ₂ readings		Deviation from mean of pair	CO ₂ Concentration
	mmHg		%	mM/l
1. 8 Feb 73	I	237.4	±0.06	30.51
	II	237.3		
	Mean	237.4		
2. 15 Feb 73	I	232.6	±0.09	29.91
	II	233.0		
	Mean	232.8		
3. 22 Feb 73	I	236.0	±0.13	30.36
	II	236.6		
	Mean	236.3		
4. 15 Mar 73	I	232.8	±0.17	29.99
	II	234.0		
	Mean	233.4		
5. 29 Mar 73	I	239.7	±0.00	30.80
	II	239.7		
	Mean	239.7		
6. 12 Apr 73	I	231.4	±0.04	29.85
	II	231.6		
	Mean	231.5		
7. 13 Apr 73	I	230.4	±0.56	29.87
	II	233.0		
	Mean	231.7		
8. 30 May 73	I	232.3	±0.09	29.90
	II	232.7		
	Mean	232.5		
Mean (X̄)				30.14mM
Standard Deviation (SD)				±0.23
Coefficient of Variation (C)				±0.77%

Table 3. Preliminary experiment testing the efficacy of using evacuated test tubes for blood gas analysis. Whole blood sample, N=3

Condition			pH (units)	P _{CO₂} (mmHg)	P _{O₂} (mmHg)	Actual HCO ₃ ⁻ (mM/l)	% Sat (%)
1. Transferred directly from vein into syringe	$\bar{X}$		7.356	45.7	29.5	24.4	53.2
	SD		.018	1.5	7.1	2.0	14.1
	SE		.006	.7	2.4	.7	4.5
2. Transferred from syringe to test tube containing mineral oil	$\bar{X}$		7.364	46.2	35.0	25.2	65.5
	SD		.021	1.6	6.5	2.1	15.1
	SE		.007	.8	3.2	.7	5.0
	P		N.S.	N.S.	N.S.	N.S.	N.S.
3. Transferred to Vacutainer [®] from syringe	$\bar{X}$		7.388	34.8	77.0	20.0	94.5
	SD		.028	2.8	13.5	4.1	18.5
	SE		.010	1.0	4.5	1.3	6.4
	P		N.S.	<.001	<.001	<.05	<.001

Mean duplicate samples, however, were found to be ± 0.72 SD for serum and ± 0.98 SD for red cells indicative of an intersample variance.

pK₁' values for serum and cells obtained in this study are compared with representative data found in the literature (Table 6). pK_s' in the cited references were all determined by a manometric technique and the pK_s' values are in agreement within experimental error.

A direct comparison of pK_c' obtained by different workers is complicated because of the different technical

approaches that were used for the derivation of pK_c'. For example: data of Dill, Daly and Forbes was uncorrected for HbCO₂; Deane and Smith determined CO₂ content of plasma and whole blood, and by a series of calculations involving CO₂ content and hematocrit they indirectly calculated pK_c' values. Bauer and Schroeder determined pK_c' by calculating [HCO₃]_c and [HbCO₂]_c from the Donnan ratio for chloride. pK_c' of the present study is approximately in the midrange of the 1957 and 1972 investigations.

All of the pK_c' values in Table 6 are based upon electrometric pH_c of erythrolysates obtained by hemolyzing

Table 4. Summation of whole blood indices. Samples were analyzed as drawn from the body prior to centrifugation.
Sample size, N=10

	MEAN	S.D.
Hct (%)	46.0	3.6
pH (U)	7.360	.030
P _{CO₂} (mmHg)	43.8	6.1
P _{O₂} (mmHg)	37.0	11.2
Hb (gm%)	15.3	.7
HbO ₂ (% sat)	68.2	14.2
HbCO _{NS} * (% sat)	3.4	.9
HbCO _S * (% sat)	10.0**	2.0
H ₂ O CONTENT (gm/gm)	.7831	.0259
T _{CO₂} (mM/l)	23.71	2.00
T _{O₂} (mM/l)	6.76	1.77
H ₂ CO ₃ (mM/l)	1.35	.19
HCO ₃ ⁻ (mM/l)	22.36	2.14
pK ₁ '	6.147	.043

NS* - non-smoker (N=5)

S* - smoker (N=5)

** P < .001 from non-smokers

Table 5. Data utilized for calculation of plasma and cell pK_1' . Sample size, $N=10$

Condition	pH U	P_{CO_2} mmHg	T_{CO_2} mM/l	H_2O cont. gm/gm	H_2CO_3 mM/l	$HbCO_2$ mM/l	HCO_3^- mM/l
1. SERUM AD O R	$\bar{X}$	43.9	26.53	.9103	1.35	*	25.18
	SD	4.4	1.20	.0037	.05		1.11
	$\bar{X}$	37.4	22.40	.9112	1.15	*	21.25
	SD	2.5	.28	.0087	.03		.41
	$\bar{X}$	39.2	25.29	.9117	1.20	*	24.09
	SD	3.6	.38	.0061	.02		.34
2. RBC AD O R	$\bar{X}$	46.0	17.14	.6696	1.20	1.97	13.97
	SD	4.7	1.57	.0173	.05	.37	1.09
	$\bar{X}$	38.6	13.02	.6817	1.00	1.18	10.84
	SD	1.8	.50	.0237	.01	.15	.58
	$\bar{X}$	41.2	16.86	.6815	1.07	3.00	12.79
	SD	1.5	.35	.0238	.02	.27	.43

* $HbCO_2$ for serum was assumed to be negligible.

Table 6. Representative human pK_1' values in the literature

Author	Sample	Subjects	Mean pK_1'	Standard Deviation	Coefficient of Variation (%)
Dill, Daly and Forbes (1937)	serum, 0 ¹	2	6.113	.016	.26
	cells, 0	1	6.056	.036	.59
	cells, R ²	2	5.982	.034	.57
Deane and Smith (1957)	serum, AD ³	11	6.11	.03	.49
	cells, AD	11	6.18	.06	.97
Bauer and Schroeder (1972)	serum, 0	*	6.11	.02	.33
	cells, 0	*	6.10	.02	.33
	serum, R	*	6.11	.01	.16
	cells, R	*	6.12	.02	.33
Present Study (1974)	serum, AD	10	6.099	.028	.46
	cells, AD	10	6.098§	.039	.64
	serum, 0	10	6.125	.026	.43
	cells, 0	10	6.151§	.040	.65
	serum, R	10	6.105	.026	.42
	cells, R	10	6.121§	.032	.52

0¹ oxygenatedR² reducedAD³ as drawn

* number of subjects not reported

§ add 0.036 to correct for red cell junction potential error (Siggaard-Anderson, 1961)

red cells. Siggaard-Anderson has pointed out the possibility of a red cell junction potential error arising from the hemolyzed cell upon the pH sensitive glass. He determined a correction factor of $\pm 0.036 \pm 0.005$ SD for pH_c . A correction of this type would effectively increase pK_c' by the same degree, i.e., ± 0.036 (Siggaard-Anderson, 1961).

Table 7 presents pK_c' values determined from our data by the same procedure used by Bauer and Schroeder. The pK_c' obtained by this method are slightly greater than that obtained by the direct method (Table 6). The slightly greater pK_c' is due primarily to the greater value of HbCO_2 calculated from (3) of Table 7. A comparison of $[\text{HbCO}_2]_c$ calculated by both methods is shown in Table 7. It should be remembered that in the present investigation $[\text{HbCO}_2]_c$ was calculated per (3) of Table 7, however, the $[\text{HCO}_3]_c$ was directly determined manometrically. Agreement by these two totally different methodologies lends credence to the derived pK_c' value. As mentioned, Table 7 also present data showing the calculation of carbamino hemoglobin content of cells by two methods. Bauer and Schroeder (1972) state that by using the equation presented by Stadie and Hawes (1928) - which are based upon hemoglobin solutions, would overestimate HbCO_2 content. However, the earlier graphs of Ferguson and Roughton (1937) do not overestimate $[\text{HbCO}_2]_c$, in fact Bauer and Schroeder's values appear to be slightly greater.

Donnan distribution ratios of aH^+ , Cl^- , and HCO_3^- are presented in Table 8. For comparative purposes,

distribution ratios calculated from regression equations of Fitzsimons and Sendroy (1961) are also presented. There is close agreement of ${}^r\text{Cl}^-$ and ${}^r\text{HCO}_3^-$ with the Fitzsimons and Sendroy study. The ${}^r\text{aH}^+$ of the present study has been calculated with the pH_c corrected for the red cell junction potential error, this would explain the greater range of ${}^r\text{aH}^+$ as compared to Fitzsimons and Sendroy.

DISCUSSION

The Theoretical and Experimental Determination of pK_1' .

The Debye and Huckel theory of ionic interaction of solutions was formulated in 1923. The extension of the theory to biological systems which are composites of water, protein, salts, and non-electrolytes is complicated and presents theoretical and practical difficulties.

Vapor pressure, boiling point, osmotic pressure, freezing point, membrane equilibria, and other properties of very dilute aqueous solutions of non-electrolytes and electrolytes may be explained by the application of the laws for ideal solution and the assumption that the (strong) electrolytes are completely ionized. The Debye and Huckel theory evaluates the electrical effect of the charges of all the ions in the solution upon a given ion, A; in terms of the valence and concentration of all the ions; the valence, concentration, the effective ionic diameter of the given ion; and the dielectric constant of the solution. Although in more concentrated

Table 7. Derivation of pK_c' by method used by Bauer and Schroeder (1972)

1. Determine Donnan distribution ratio for chloride, r_{Cl^-} .

2. Determine $[HCO_3]_c$:

$$(ii) \quad [HCO_3]_c = r_{Cl^-} \times [HCO_3^-]_s \times \frac{H_2O_c}{H_2O_s}$$

3. Determine $[HbCO_2]_c$:

$$(iii) \quad [HbCO_2]_c = [T_{CO_2}]_c - [HCO_3^-]_c - [H_2CO_3]_c$$

4. Derive pK_c' :

$$pK_c' = pH_c - \log \frac{[T_{CO_2}]_c - [HbCO_2]_c - [H_2CO_3]_c}{[H_2CO_3]_c}$$

	AD	0	R
pK_c'	6.114	6.164	6.132

Calculation of $[HbCO_2]_c$ content

Method:	Bauer and Schroeder (1972)	Ferguson and Roughton (1936)
AD	2.49	1.97
O	1.50	1.18
R	3.35	3.00

Table 8. Donnan distribution ratios of hydrogen ion activity, chloride, and bicarbonate, N=10

		r_{aH^+}	r_{Cl^-}	$r_{HCO_3^-}$
As Drawn	$\bar{X}$	.698 (.651)*	.726 (.703)	.745 (.702)
	SD	.042	.136	.072
Oxygenated	$\bar{X}$	.682 (.630)	.678 (.665)	.699 (.681)
	SD	.037	.115	.075
Reduced	$\bar{X}$	.676 (.642)	.724 (.715)	.710 (.681)
	SD	.038	.148	.068

* For comparative purposes, values in parentheses are distribution ratios obtained from the regression equations of Fitzsimons and Sendroy (1961)

solutions the departure from the ideal state is great, the Debye-Huckel equation is useful and may be written as:

$$(iv) \log_{10} \gamma_i = - \frac{\beta_o \sqrt{\Gamma}}{1 + B a \sqrt{\Gamma}} + G \Gamma$$

$$\beta_o = \frac{1.8 \times 10^6}{D_o^{3/2} T^{3/2}} \quad (1.8 \times 10^6 \text{ calculated from universal constants})$$

D_o = dielectric constant of water

T = absolute temperature

$$\beta_o = 0.505 \text{ at } 25^\circ$$

$$\beta_o = .532 \text{ at } 38^\circ$$

$B = 50.4 a \times 10^8$ (50.4×10^8 is calculated from universal constants)

$$B_{25^\circ} = 0.328a \times 10^8$$

$$B_{38^\circ} = 0.33a \times 10^8$$

G is a function which gives the variation of the dielectric constant of the solvent with Γ . $\Gamma = 1/2 \sum Z^2 [c]$; i.e. $1/2$ the summation of the product of the concentration, $[c]$, (in moles per liter) of each ion by its valence. Z squared, is the ionic concentration. γ_i is the activity coefficient of the ion. a = mean ionic collision sphere or distance in cm of closest approach between the center of the ion A and the center of any surrounding positive or negative ion.

Using an alternative form of this equation, Hastings and Sendroy (1925) showed that in water, the relation of pK_1 , pK_1' , β_0 , and $\sqrt{\Gamma}$ at 38°C is:

$$(v) \quad pK_1' = pK_1 - \beta_0 \sqrt{\Gamma}$$

$pK_1 = 6.22$ from which,

$$(vi) \quad \log HCO_3 = -\beta_0 \sqrt{\Gamma}$$

β_0 was found to be 0.54 which was practically the theoretical value, 0.532. Recent work by Fitzsimons and Sendroy (1961) recalculated pK_1 at 37°C to be 6.318; in another careful study, Siggaard-Anderson (1962) independently arrived at a figure of $pK_1 = 6.324$ at 37°C.

Stadie (1928) reported that a similar relation also exists for hemoglobin solutions.

An important point, which seems to have been missed by some workers when calculating the theoretical pK_1' of cells, has been the relation of Γ and μ . The equation

$$(vii) \quad \frac{\Gamma_i}{[c_i]} = \frac{\mu_i}{(c_i)}$$

gives the relation between Γ the ionic strength per liter and μ the ionic strength per kilo of solvent. It is important to note that the Debye-Huckel equation requires that the ionic strength be expressed as moles per liter, i.e., Γ . Since

$$(viii) \quad \Gamma = 1/2 \sum Z^2 [c_i] = 1/2 [W] \sum Z^2 (c_i) = [W] \mu$$

equation (viii) becomes on substitution and rearrangement

$$(ix) \quad \log \gamma_i = -0.53 \sqrt{[W] \mu} \quad (\text{Stadie, 1928}).$$

It is important to realize that the use of equation (ix) without the $[W]$ factor leads to erroneous results in concentrated protein solutions; e.g., in the erythrocyte $[W] = 0.65$ and $\mu = 0.170$ moles per kilo of H_2O whereas for serum $[W] = 0.93$ and $\mu = 0.150$. Failure to incorporate $[W]$ would tend to underestimate pK_1' .

An additional factor exists which complicates the experimental results of earlier workers thereby further precluding agreement of their experimental pK_1' values with that obtained theoretically. Van Slyke and coll. (1923) assumed that the high protein content of the cell fluid did not affect the potential readings of their electrometric pH electrode. Recent evidence has shown a red cell junction potential effect upon the pH sensitive glass of approximately -0.036 pH units (Siggaard-Anderson, 1961). Therefore, results of Van Slyke and coll. (1925) and those of Dill, Daly, and Forbes (1937) based on electrometric pH would necessarily be lower than the theoretical values.

The theoretical pK_1' at 37°C calculated with constants derived and reported by Siggaard-Anderson (1962) and $[W]$ obtained in the present study may be calculated as:

$$pK_1' = pK_1 - B \sqrt{[W] \mu}$$

$$pK_1' = 6.324 - 0.495 \sqrt{(0.677) (0.170)}$$

$$= 6.156$$

This value for pK_C' may be compared with those presented in Table 6. It should be noted that when the red cell junction potential correction for pH_C is made, pK_C' values for the AD, O, and R samples are 6.134, 6.187, and 6.157 respectively. This agreement, for the first time, of experimental with the theoretical pK_C' is due to the increased precision afforded by the incorporation of pH_C and $HbCO_2$ corrections which were previously unknown factors.

The Donnan Distribution Ratio

In a classical investigation, Van Slyke, Wu, and McLean (1923) formulated the laws governing the electrolyte and water distribution between cells and serum. Donnan distribution ratios were shown, as a first approximation, to be expressed by the equation:

$$(x) \quad r = \frac{[aH^+]_s}{[aH^+]_c} = \frac{[Cl^-]_c}{[Cl^-]_s} = \frac{[HCO_3^-]_c}{[HCO_3^-]_s}$$

Their experimentally determined distribution ratios were found to be related as follows:

$$(xi) \quad \frac{[aH^+]_s}{[aH^+]_c} = 0.77 \frac{[Cl^-]_c}{[Cl^-]_s} =$$

$$0.62 \frac{[HCO_3^-]_c}{[HCO_3^-]_s}$$

aH^+ represents pH electrometrically determined,

$[Cl^-]$ represent molality in mM/kilo. H_2O ,

$[HCO_3^-]$ represent molality in mM/kilo H_2O nor corrected for carbamate.

Fitzsimons and Sendroy (1961) using improved methods have reported distribution ratios bearing the relationship of

$$(xii) \quad \frac{[aH^+]_s}{[aH^+]_c} = 0.92 \frac{[Cl^-]_c}{[Cl^-]_s} =$$

$$\frac{0.93 [HCO_3^-]_c}{[HCO_3^-]_s}$$

where $[HCO_3^-]_c$ was carbamate corrected. Similar relationships were also shown by Dill, Edwards, and Consolazio (1937) and also by Deane (1959).

On the basis of data presented in Table 8 incorporating carbamate adjusted $[HCO_3^-]_c$ and pH_C with the red cell junction potential correction, the distribution of diffusable ions in serum and cells related to the non-diffusible ions is shown to be:

$$(xiii) \quad \frac{[aH^+]_s}{[aH^+]_c} = 0.97 \frac{[Cl^-]_c}{[Cl^-]_s} =$$

$$0.96 \frac{[HCO_3^-]_c}{[HCO_3^-]_s}$$

Similarly, if Fitzsimons and Sendroy's aH^+ values were also corrected for red cell junction potential error the Cl^- and HCO_3^- ratios would closely approximate unity. As the data are improved, the validity of the Donnan relationship governing the distribution of H^+ , Cl^- , and

HCO₃⁻ ions between serum and electrocytes is reinforced.

A Simplified Method for the Calculation of Cell Bicarbonate

A primary objective of this investigation has been to obtain a reliable pK_c' from which it would then be possible to incorporate pH_c and P_{CO}₂ into the Henderson-Hasselbalch equation thereby obtaining a carbamate corrected [HCO₃]_c.

To illustrate, the constants for oxygenated pK_c' = 6.187 and α_c = 0.026 would be used and the three variables: pH_c', P_{CO}_{2c}, and [HCO₃]_c would then be related by the equation,

$$(xiv) \text{ pH}_c = 6.187 + \log \frac{[\text{HCO}_3]_c}{0.026 \text{ P}_{\text{CO}_2}}$$

An example is presented:

1st step - Determine P_{CO}_{2c} =
38.6 mmHg

Determine, pH_c =
7.186
+ 0.036 (correction for
7.222 junction po-
tential)

2nd Step - Calculate bicarbonate concentration,

$$7.222 = 6.187 + \log \frac{[\text{HCO}_3]}{0.026(38.6)}$$

$$1.035 = \log \frac{[\text{HCO}_3]}{1.00}$$

Antilog₁₀ of 1.035 is 10.84; hence

$$10.84 = \frac{[\text{HCO}_3^-]}{1.00}$$

$$[\text{HCO}_3^-] = 10.84 \text{ mM/l}$$

Although this method greatly simplifies the determination of [HCO₃]_c it still necessitates determination of cell indices and the use of various correction factors. An attempt was made to further simplify the method.

The Henderson-Hasselbalch equation may be rearranged and alternately be presented in the following manner:

$$(xv) \log \text{HCO}_3 = \log (\alpha \times \text{P}_{\text{CO}_2}) + \text{pH} - \text{pK}_1'$$

In this form, it is obvious that, +pH -pK₁', is simply a mathematical relationship of a difference term. Therefore the difference of the pH corrected factors; i.e., 7.222 - 6.187 = 0.035 or of the pH uncorrected terms 7.186 - 6.151 = 0.035 are identical.

A further simplification would be to use a regression equation that would only necessitate the direct determination of pH_s (or the pH of whole blood plus 0.010 pH units to correct for the red cell junction potential for whole blood, Severinghaus and coll., 1956), and calculate the resulting pH_c. A regression equation for the determination of intra-erythrocytic pH values from pH_s has been determined from the data of this investigation and is reported as:

$$(xvi) \text{ pH}_c = 0.796 \text{ pH}_s + 1.301$$

One may also assume $P_{CO_{2s,b}} = P_{CO_{2c}}$. Thereby it would be possible to analyze either a whole blood or serum sample for pH and P_{CO_2} and insert the derived values into equation (xiv) to obtain bicarbonate concentration of cells corrected for carbamino hemoglobin.

An illustrated example is presented for this method:

$$(xvii) \text{ pH}_c = 6.151 + \frac{\log [\text{HCO}_3^-]_c}{0.026 P_{CO_{2c}}}$$

1st Step - Determine, $P_{CO_{2s}} = 37.4$
mmHg = $P_{CO_{2c}}$

Determine, pHs = 7.392

Calculate, pHc

$$\begin{aligned} \text{pH}_c &= 0.796 \text{ pHs} + 1.301 \\ &= 0.796 (7.392) + 1.301 \\ &= 7.186 \end{aligned}$$

2nd Step - Calculate bicarbonate concentration,

$$7.186 = 6.151 + \log \frac{[\text{HCO}_3^-]}{(0.026) (37.4)}$$

$$1.035 = \log \frac{[\text{HCO}_3^-]}{0.972}$$

antilog 1.035 is 10.84; hence

$$\begin{aligned} 10.84 &= \text{HCO}_3^- / 0.972 \\ \text{HCO}_3^- &= 10.54 \text{ mM/l} \end{aligned}$$

As seen from the first calculation, this value is 0.30 mM/l less than the directly determined, carbamate corrected, cell bicarbonate concentration. The percent error would be $0.30 / 10.84 \times 100 = 2.7\%$ whereas the coefficient of variation for the directly determined $[\text{HCO}_3^-]$ is $0.58 / 10.84 \times 100 = 5.4\%$. This result would be well within experimental error.

The indirect method involving simply the determination of either whole blood (if the correction factor of +.01 pH units were appended) or serum pH and P_{CO_2} and the use of the regression equation to determine pH_c greatly simplifies the determination of $[\text{HCO}_3^-]_c$ for normal subjects utilized in this study.

Whether the same relationship would be upheld in diseased states remains to be investigated. Evidence does exist that the distribution ratio of ions between cells and serum is upheld under conditions varying widely from normal (Fitzsimons and Sendroy, 1961). The evidence that diseased states may alter pK_s' (Trenchard and coll., 1968) has not been substantiated by other workers in the field (Sinclair and coll., 1968; Cullen and coll., 1925; Robinson and coll., 1934; Austin and coll., 1968).

In summary:

1) pK_1' was determined for human serum from venous blood and analyzed in the as drawn, oxygenated, and reduced state and found to be 6.099, 6.125, and 6.105, respectively.

2) pK_1' was determined for human erythrocytes from venous blood and

analyzed in the as drawn, oxygenated and reduced state and found to be 6.098, 6.151, and 6.121, respectively, when uncorrected for pH_c red cell junction potential error;

3) when corrected for junction potential error, pK_c' values are reported as 6.144, 6.187, and 6.157 for the as drawn, oxygenated, and reduced state;

4) theoretical pK_c' was calculated from the relation $pK_1' = pK_1 - B \sqrt{[W] \mu}$ and found to be 6.156, where pK_1 (6.324 at 37°C) is the "thermodynamic" dissociation exponent. B (0.495) is a constant derived theoretically, W (.68) is the fraction of cell water, and μ (.170) is the ionic strength of erythrocytes;

5) the relation among the distribution ratio, r , with serum pH was found to be

$$r = \frac{[aH^+]_s}{[aH^+]_c} = 0.97 \frac{[Cl^-]_c}{[Cl^-]_s} = 0.96 \frac{[HCO_3^-]_c}{[HCO_3^-]_s} ;$$

6) a simplified method of calculating carbamate corrected, cell bicarbonate concentration using the determined cell pK_1' value has been formulated.

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UNCLASSIFIED

Security Classification

DOCUMENT CONTROL DATA - R & D

(Security classification of title, body of abstract and indexing annotation must be entered when the overall report is classified)

1. ORIGINATING ACTIVITY (Corporate author)		2a. REPORT SECURITY CLASSIFICATION																										
NAVAL SUBMARINE MEDICAL RESEARCH LABORATORY Naval Submarine Medical Center		Unclassified																										
3. REPORT TITLE		2b. GROUP																										
FIRST APPARENT DISSOCIATION CONSTANT OF CARBONIC ACID, pK_1' , IN HUMAN SERUM AND RED CELLS																												
4. DESCRIPTIVE NOTES (Type of report and inclusive dates)																												
Interim report																												
5. AUTHOR(S) (First name, middle initial, last name)																												
Arthur A. Messier																												
6. REPORT DATE		7a. TOTAL NO. OF PAGES	7b. NO. OF REFS																									
20 March 1974		22	30																									
8a. CONTRACT OR GRANT NO.		9a. ORIGINATOR'S REPORT NUMBER(S)																										
b. PROJECT NO.		NSMRL Report No. 780																										
c. MR041.01.01-0125B9XX		9b. OTHER REPORT NO(S) (Any other numbers that may be assigned this report)																										
d.																												
10. DISTRIBUTION STATEMENT																												
Approved for public release; distribution unlimited																												
11. SUPPLEMENTARY NOTES		12. SPONSORING MILITARY ACTIVITY																										
		Naval Submarine Medical Rsch Lab Box 900, Naval Submarine Base Groton, Connecticut 06340																										
13. ABSTRACT																												
The first apparent dissociation constant of carbonic acid, pK_1' of serum and red cells was determined on venous blood of ten healthy, adult, male, human subjects. pH and PCO_2 of serum and red cells were analyzed electrometrically and a micromanometric method was used for the determination of total carbon dioxide content. Erythrocyte carbamino hemoglobin levels were estimated and used for the correction of erythrocyte pK_1'. Each blood sample was subjected to the following regimen before centrifugation, 1) <u>As Drawn</u> from the antecubital vein, 2) <u>Oxygenated</u> with a 5% CO_2, O_2 balance gas mixture, and 3) <u>Reduced</u> with a 5% CO_2, N_2 balance gas mixture, pK_1' of serum and red cells are presented: <table border="1"> <thead> <tr> <th></th> <th></th> <th>AS DRAWN</th> <th>OXYGENATED</th> <th>REDUCED</th> </tr> </thead> <tbody> <tr> <td>SERUM</td> <td>Mean</td> <td>6.099</td> <td>6.125</td> <td>6.105</td> </tr> <tr> <td>(N=10)</td> <td>SD</td> <td>.028</td> <td>.026</td> <td>.026</td> </tr> <tr> <td>RBC</td> <td>Mean</td> <td>6.144</td> <td>6.187</td> <td>6.157</td> </tr> <tr> <td>(N=10)</td> <td>SD</td> <td>.039</td> <td>.040</td> <td>.032</td> </tr> </tbody> </table> The consistently larger values for red cell pK_1' than the respective serum data may be attributive of the greater amount of carbamino hemoglobin concentration present in the erythrocytes. A simplified method for the calculation of a, carbamate corrected, erythrocyte bicarbonate concentration using the experimentally determined red cell pK_1' value has been formulated. The method involves the use of a regression equation relating serum and red cell pH, the equivalence of serum and red cell PCO_2, along with experimentally determined red cell pK_1'.						AS DRAWN	OXYGENATED	REDUCED	SERUM	Mean	6.099	6.125	6.105	(N=10)	SD	.028	.026	.026	RBC	Mean	6.144	6.187	6.157	(N=10)	SD	.039	.040	.032
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S/N 0102-014-6600

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(PAGE 2)

Security Classification