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THE REACTION OF MERCURIC CHLORIDE WITH THE UNREACTIVE SULPHYDRYL GROUPS OF HEMOGLOBIN

by

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B.Sc. University of Manitoba, 1964

A THESIS SUBMITTED IN PARTIAL FULFILLMENT

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March 25, 1976.

(date)

ABSTRACT

The reaction of $HgCl_2$ with the unreactive -SH groups of hemoglobin was investigated using amperometric techniques to determine the reaction stoichiometry and kinetics. Mercuric chloride was found to react with -SHβ112 and -SHα104 in neutral solution with a constant one to one stoichiometry regardless of the ambient halide salt concentration. As well as reacting with the three pairs of sulphydryl groups SHβ93, SH-β112, SH-α104, $HgCl_2$ was weakly bound by HbA at an unidentified locus to the extent of 2 molar equivalents per tetramer.

The apparent second order rate constants for the reactions of -SHβ112 and -SHα104 were sensitive to pH, ionic strength, hemoglobin concentration, ligand state of the heme, and to the presence of divalent cations. It was observed, however, that the measured diffusion constant for $HgCl_2$ in hemoglobin solution was dependent on hemoglobin concentration, ligand state of the heme, the presence of divalent cations, and to some extent on the ambient salt concentration. When the apparent rate constants were corrected for this factor it was found that the second order rate constants were more or less independent of ligand state and hemoglobin concentration.

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LIST OF SYMBOLS AND ABBREVIATIONS

Symbols

a b	initial concentrations of a chemical species
C	concentration of ionic species
D	concentration of dimeric hemoglobin
D_o	diffusion coefficient of an ion
D_{Hg}	diffusion coefficient of mercuric ion
e	reaction endpoint
Hb	instantaneous tetramer concentration
HBT	total hemoglobin concentration
Hg	instantaneous Hg^{+2} concentration
HGT	total mercury concentration
HgHb	concentration of the mercury-hemoglobin reaction product
$HgCl_x$	concentration of species x 1, 2, 3, or 4
I	current, amps
i_{lim}	limiting current of a polarographic wave
k	rate constant
K_{app}	apparent rate constant
K^1	arbitrary constant
K_i	dissociation constant for $Hb_4 \rightleftharpoons Hb_2$
M	hemoglobin monomer concentration
n	charge on ion
Φ	inversely proportional to D/HBT
Res	residual HGT-HBT
S	sensitivity, amps.mole^{-1}

T	temperature; ^{o}C
t	time; sec
θ	ratio of HGT/$HgCl_x$
x	amount of mercuric ion reacted
X_o, X_1	data points on a recorded reaction curve
y	amount of hemoglobin dimer reacted
z	unknown exponent

Abbreviations

α, β, γ	hemoglobin subunits with different amino acid sequences
$\alpha_1\beta_1$, $\alpha_1\beta_2$	hemoglobin dimers
E_a	energy of activation
Hb, Hb_4, Hb_2	hemoglobin, Hb tetramer, Hb dimer
HbA	human hemoglobin
HbE	equine hemoglobin
HbF	foetal hemoglobin
MetHb	methemoglobin
Rate I	the reaction of the first two equivalents of $HgCl_2$ with Hb_4
Rate II	the reaction of the second two equivalents of $HgCl_2$ with Hb_4
Rate III	the reaction of the third two equivalents of $HgCl_2$ with Hb_4
-SHβ112, -SHβ93, -SHα104	sulphydryl groups in various hemoglobin subunits, located at the position in the amino acid sequence indicated by the number
S.C.E.	Standard Calomel Electrode

INTRODUCTION

The reaction of protein sulphydryl groups with heavy metal reagents, particularly mercury compounds, was an important assay technique several decades ago. Recently the reaction has been increasingly involved with various probes of native protein structure (1-9). One such probe is based on the rate of halide exchange on a mercury atom which has been reacted to a protein sulphydryl group (2). The halide exchange rate measured with a Nuclear Magnetic Resonance spectrometer, reflects steric conditions in the locale of the sulphydryl groups.

It was in connection with the particular probe described above, that I began an investigation of the stoichiometry of the reaction of mercuric chloride with human hemoglobin. The conclusions of this investigation were that not only does the cysteine group at position β93 react with mercuric ion as has previously been established (10,11), but further, the two remaining cysteine groups at α104 & β112 also react at finite rates in neutral solutions. The latter observation had been reported (12) but the conditions under which the reaction took place had never been clarified.

The sulphydryl groups at α104 and β112 are located in the interface between the α, and β subunits of the hemoglobin tetramer. I hypothesized that if the ligand related allosteric interactions in the hemoglobin tetramer are transmitted through the $\alpha_1\beta_1$ interface, then the reaction rates of -SHα104 and -SHβ112 might be sensitive

to these interactions. Since allosteric interactions in hemoglobin are currently of considerable interest, such a probe could be quite useful.

Reaction kinetics, ligand effects, etc., were thoroughly investigated. Ligand related rate differences were observed; however after clarification of the conditions governing the reaction rates it was found that the apparent relationship between reaction rates and allosteric interactions was illusory. The rate of reactions of Hg^{+2} with the -SH groups at $\beta 112$ and $\alpha 104$ appears to be dominated by the diffusion coefficient of $HgCl_2$ in the reacting solution.

The primary conclusion of the research reported here is negative, an allosteric probe was not realized. On the other hand, in the course of the investigation a great deal of empirical information was accumulated with respect to the nature of the reaction of $HgCl_2$ with the $\beta 112$ and $\alpha 104$ -SH groups of human hemoglobin. Further, classical amperometric techniques for protein sulphydryl titration were updated and streamlined as well as being stripped off of some of the mythology surrounding reaction stoichiometry.

METHODS

Hemoglobin Preparation

All hemoglobin used in experimental studies was purified from fresh whole blood and used within seven days. Although care was taken to use fresh Hb in all crucial experiments, no alteration in the reactive properties of a given Hb sample was observed even after several months storage at 4°C.

Purification of Hb from whole blood was accomplished by a method adapted from Perutz (13) and McConnell (14). The red blood cells were first separated from the plasma by centrifugation at 10,000xg for 8-10 minutes, then washed 3 or 4 times by resuspending the pellet in 0.9% NaCl.

After the final wash the red blood cells were suspended in 1 volume of distilled water and 0.4 volumes of toluene, shaken vigorously for 5 minutes and centrifuged at 10,000xg for 20 minutes. At the end of this procedure the Hb layer was siphoned out of the centrifuge tube. Saturated NaCl solution was added to bring the total NaCl concentration up to 2%; the solution was then centrifuged at 40,000xg for 1 hour. The decanted hemoglobin solution was normally 8-12mM. heme in concentration. All procedures were carried out at 4°C.

Several experiments involved the use of hemoglobin stripped of 2,3-DPG. The method of Benesh, Benesh & Yu (15) was used without modification; batches of 25ml Hb were run through a 4cm x 30cm

column of Sephadex G25 equilibrated with 0.1 M NaCl.

Hemoglobin concentration was assayed by the "Cyanomet" technique of Wootton & Blevin (16) in which Hb is converted to methemoglobin and assayed spectrophotometrically.

An aliquot of Hb (usually 200 of 8mM Hb) was mixed with 8ml of solution A consisting of 200mg $K_3Fe(CN)_6$, 140mg KH_2PO_4, 50mg KCN per liter of H_2O. After 15 minutes 2.5ml of 0.1M $Na_2B_4O_7.10H_2O$ was added and the total volume made up to 25ml with solution A. Heme concentration was determined from the absorbance measured at 540nm in a Cary Model 14 spectrophotometer using an absorptivity of 11 x 10^3 liter $mole^{-1}$ cm^{-1} (16,17).

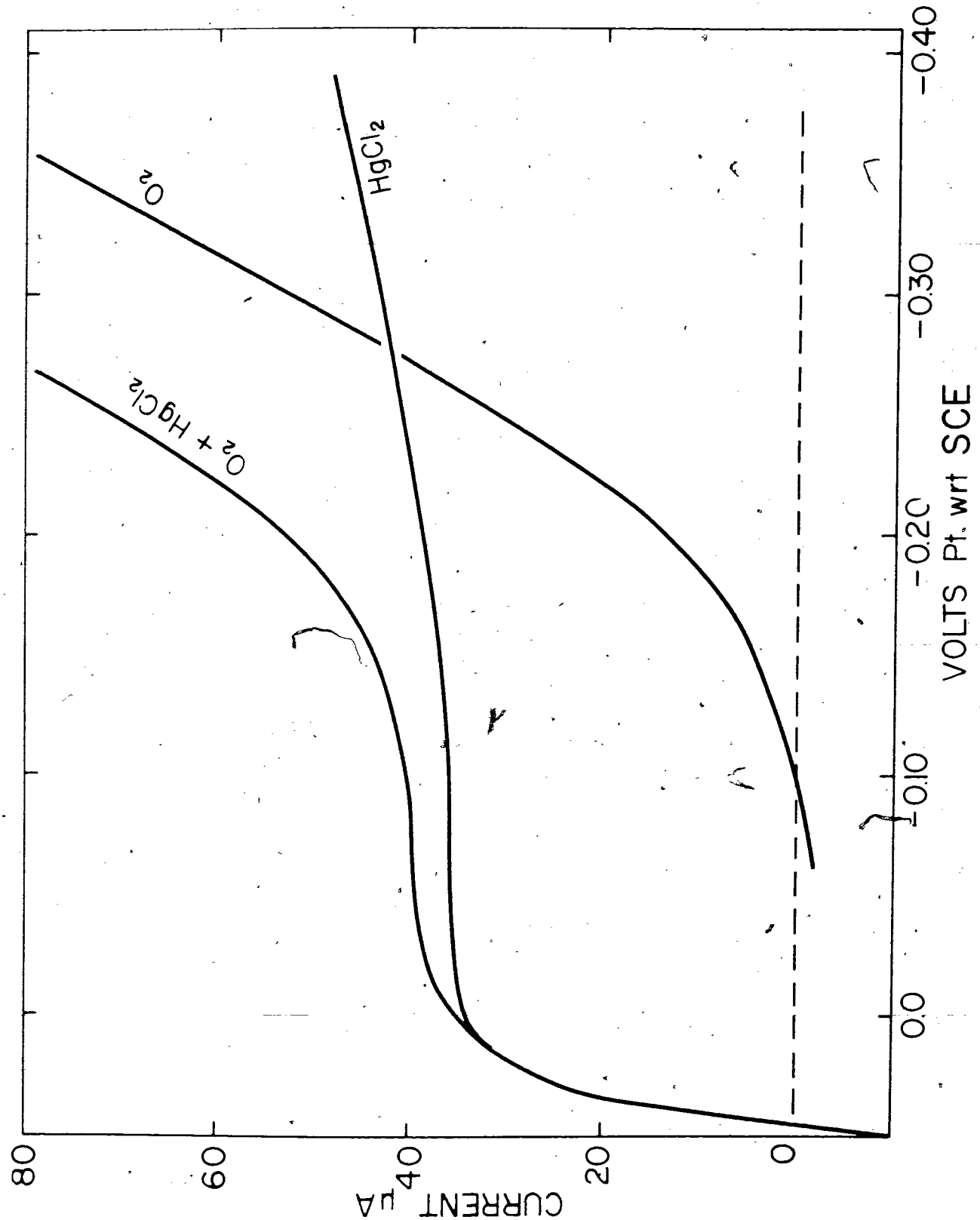
Amperometry

A mercurimetric apparatus for the titration of protein sulphydryl groups was originally described by Stricks, Kolthoff & Morren (18); the amperometric detection of Mercury (II) is reviewed by Stock (19). The techniques were used as established with a few exceptions.

a) The electrode voltage was set between 0.00 volts and -0.10 volts (platinum electrode with respect to Standard Calomel Electrode) in order that mercury could be detected in the presence of oxygen. It can be seen from the polarographic waves illustrated in Fig. 1 that this can be done without a substantial loss in sensitivity.

b) The ionic strength of the titration medium was usually much lower than the recommended 0.5M KCL. This modification will be discussed in detail.

Fig. 1. The polarographic wave for 30 μM $HgCl_2$ in oxygenated (air saturated) and deoxygenated titration medium consisting of 100 mM Phosphate, 100 mM KCl, pH 7.6. Rotating Pt electrode, in conjunction with a Standard Calomel Electrode.


80
60
40
20
0
CURRENT μA
0.0
-0.10
-0.20
-0.30
-0.40
VOLTS Pt. wrt SCE
O2
HgCl2
O2 + HgCl2

Although the electronic configuration of the amperometric apparatus differed in detail from that of Stricks et al, its functions were identical. A sensing voltage supply controlled the voltage appearing across the Pt - S.C.E. couple between 0.000 and 0.500 ± .002 volts irrespective of current. A strip chart recorder was used as current indicator.

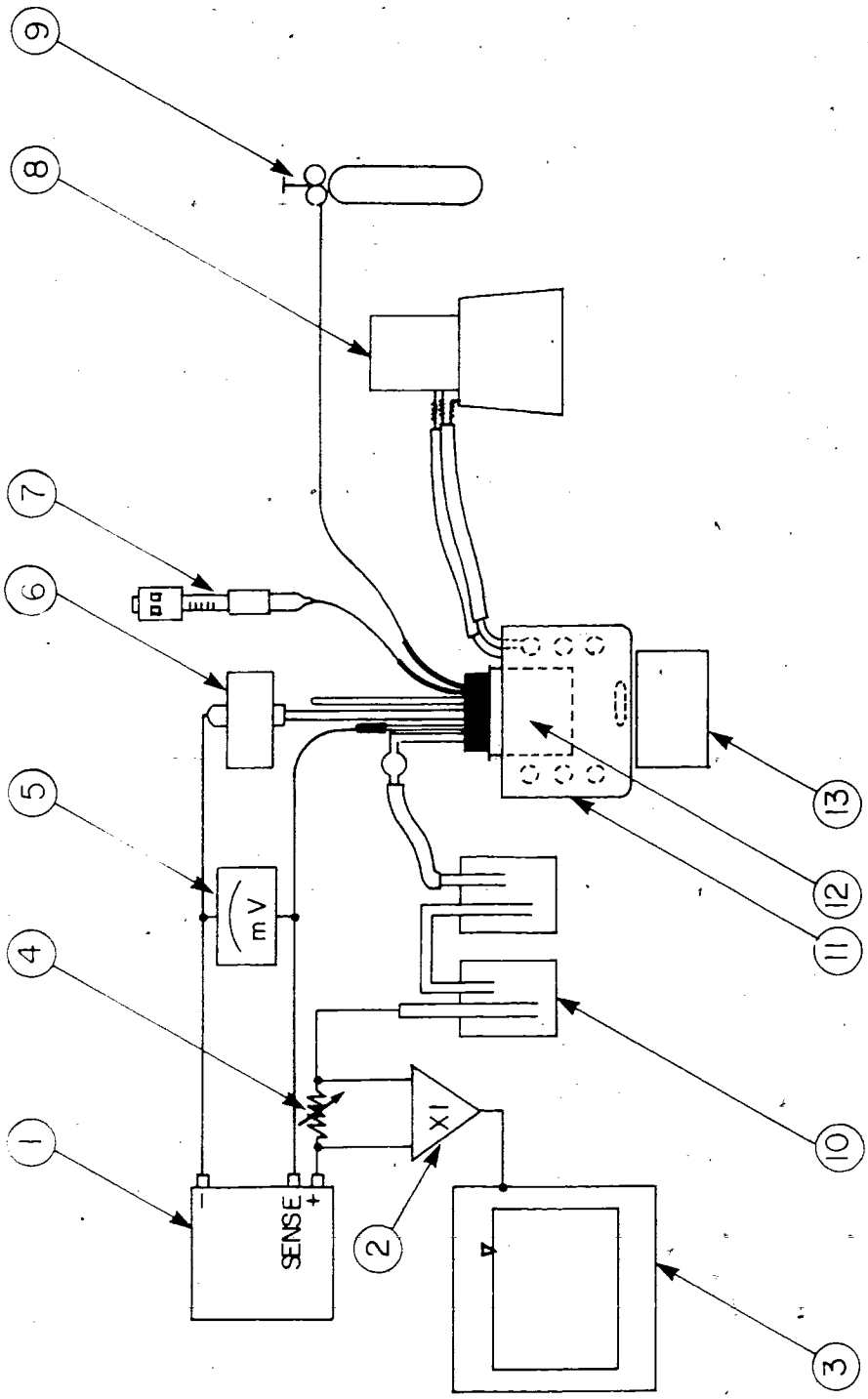
In its final configuration the mercurimetric apparatus appeared as in Figs. 2,3.

Reaction Conditions

The titration of Hb with $HgCl_2$ was carried out over a concentration range from 10 μM. heme to 300 μM. heme in titration media buffered to pH 7.6. The most commonly used medium was Buffer Ⓟ consisting of 100mM NaCl, 10mM Na_2HPO_4 adjusted to pH 7.6. Buffer Ⓒ was 2mM $CaCl_2$, 2mM $MgCl_2$ plus Buffer Ⓟ.

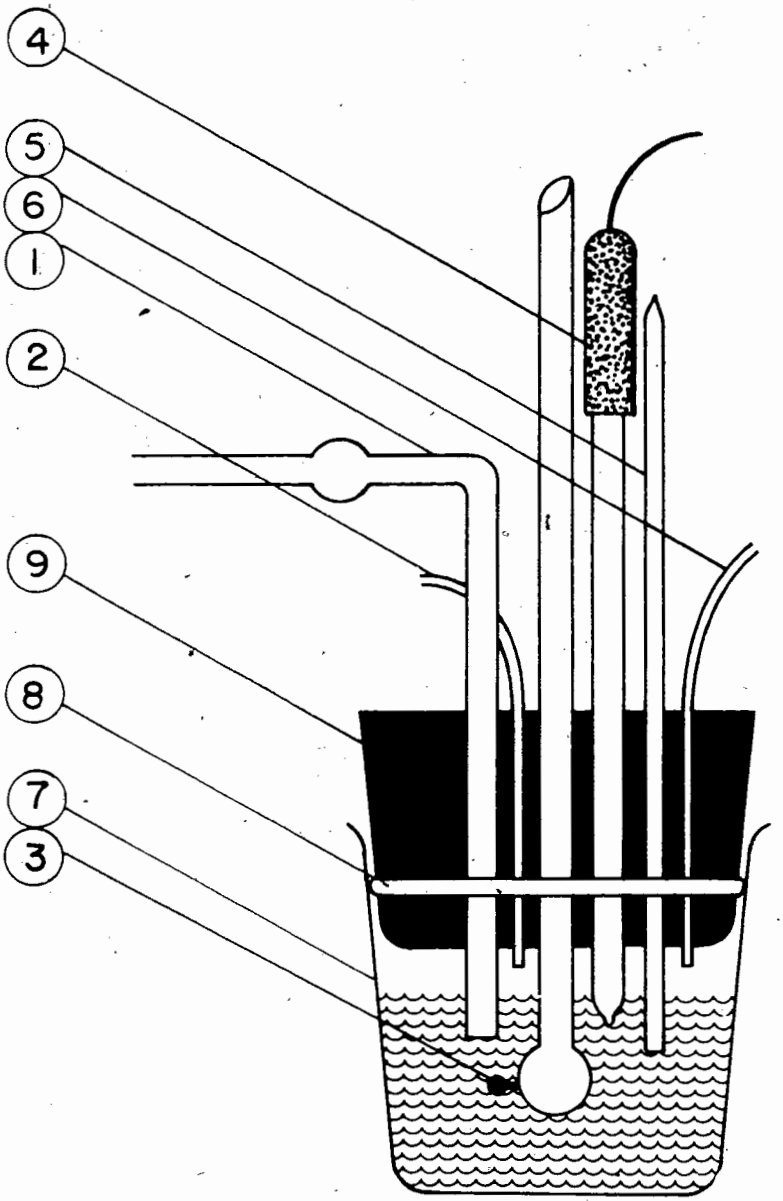
Titration of liganded Hb with $HgCl_2$ was carried out in media equilibrated with air or pure CO. For deoxy Hb the buffering medium was first deoxygenated by bubbling with High Purity N_2. After addition of the Hb aliquot the surface of the solution was swept with N_2 until the Hb was in the deoxy form. Usually this would require 20 min - 30 min. Oxygen partial pressure was monitored using the amperometric apparatus itself. By adjusting the electrode voltage to -0.35 volts Pt with respect to S.C.E. the O_2 level could be monitored down to ~1mm Hg (20) which is sufficient to deoxygenate

Fig. 2. General layout of the mercurimetric apparatus for the titration of hemoglobin -SH groups.



1. REGULATING VOLTAGE SUPPLY
2. VOLTAGE FOLLOWER
3. CHART RECORDER
4. VARIABLE RESISTANCE 1k–5k
5. VOLTMETER (CORNING 12 pH METER)
6. SYNCHRONOUS ELECTRODE ROTATOR
7. MICROMETER DRIVEN BURETTE
8. THERMOSTATED CIRCULATING WATER BATH
9. HIGH PURITY NITROGEN
10. CALOMEL MACROELECTRODE
11. WATER JACKET
12. REACTION VESSEL
13. STIRRER

Fig. 3. A detail of the reaction chamber.



1. KCL-AGAR SALT BRIDGE
2. N_2 DELIVERY TUBE
3. ROTATING PLATINUM ELECTRODE
4. STANDARD CALOMEL ELECTRODE
5. THERMOMETER
6. $HgCl_2$ DELIVERY TUBE
7. 30ml NALGENE BEAKER
8. SILICONE RUBBER GASKET
9. ACRYLIC BLOCK

normal[1] Hb A (21).

The $HgCl_2$ solution was not deoxygenated. The quantity of oxygen introduced with the $HgCl_2$ aliquot in a 50μM. heme solution would raise the O_2 partial pressure by less than 1mm Hg.

Hemoglobin aliquots were dispensed using Oxford micropipettes with disposable tips. Mercuric chloride solution was delivered to the reaction vessel by a Gilmont micrometer-driven burette through a 0.020" I.D. plastic tube. The tube was immersed in the Hb solution only during the actual delivery of $HgCl_2$ in order to avoid inaccuracies due to the diffusion of $HgCl_2$ from the tube.

The titration medium was stirred by the rotating platinum electrode. Mixing time for an aliquot of $HgCl_2$ was 1-2 seconds. The response of the amperometric apparatus at 20°C was biphasic reaching 80-90% of total deflection within 0.25 seconds and then drifting toward a final value for 10-15 seconds. The time scale for all but a few titrations was such that the lag due to finite response time was negligible.

The three different reactions (β93, β112, α104) were separated by controling the quantity of $HgCl_2$ added to the titration medium. The reaction rates for each different site differed by roughly one order of magnitude. Two different procedures were used with identical results. The solution could be pretreated with two or four

[1] Hemoglobins reacted with sulphydryl reagents at the β93 site show an increase in oxygen affinity by a factor of 3 or 4 (22-24). Riggs (22) indicates that Hb reacted with N-Ethyl malaeimide or with iodoacetimide will be ~20% oxygenated at PO_2 ~1mm Hg.

equivalents of $HgCl_2$ in order to react one or two of the -SH groups well in advance of the reaction of interest. (At least 10 times the reaction half time would be allowed.) On the other hand all of the $HgCl_2$ could be added in a single large aliquot; the rate constant would be measured only over the portion of the reaction during which the group of interest was reacting. Results from the two different methods were indistinguishable. Sensitivity was measured after each reaction (Fig. 4) by adding successive small aliquots of $HgCl_2$ to the solution.

The temperature of the titration medium was controlled using a water jacket in conjunction with a thermostated circulating water bath, and monitored with a thermometer with an accuracy of $\pm .1^{o}C$.

Data Analysis

The computation of apparent reaction rate constants was accomplished by computer using an APL program which fitted a second order rate curve to the titration data by an interative procedure (Fig. 5). Because there were ambiguities as to the initiation time for the reaction due to finite mixing time of the reagents and as to the endpoint of the reaction; the program was designed to operate without reference to these two parameters.

A typical chart record is presented in Fig. 4. Data were collected at any suitable constant time interval over a segment of the curve isolated from the extrema of the reaction. Normally

Fig. 4. Typical chart record for a hemoglobin titration.

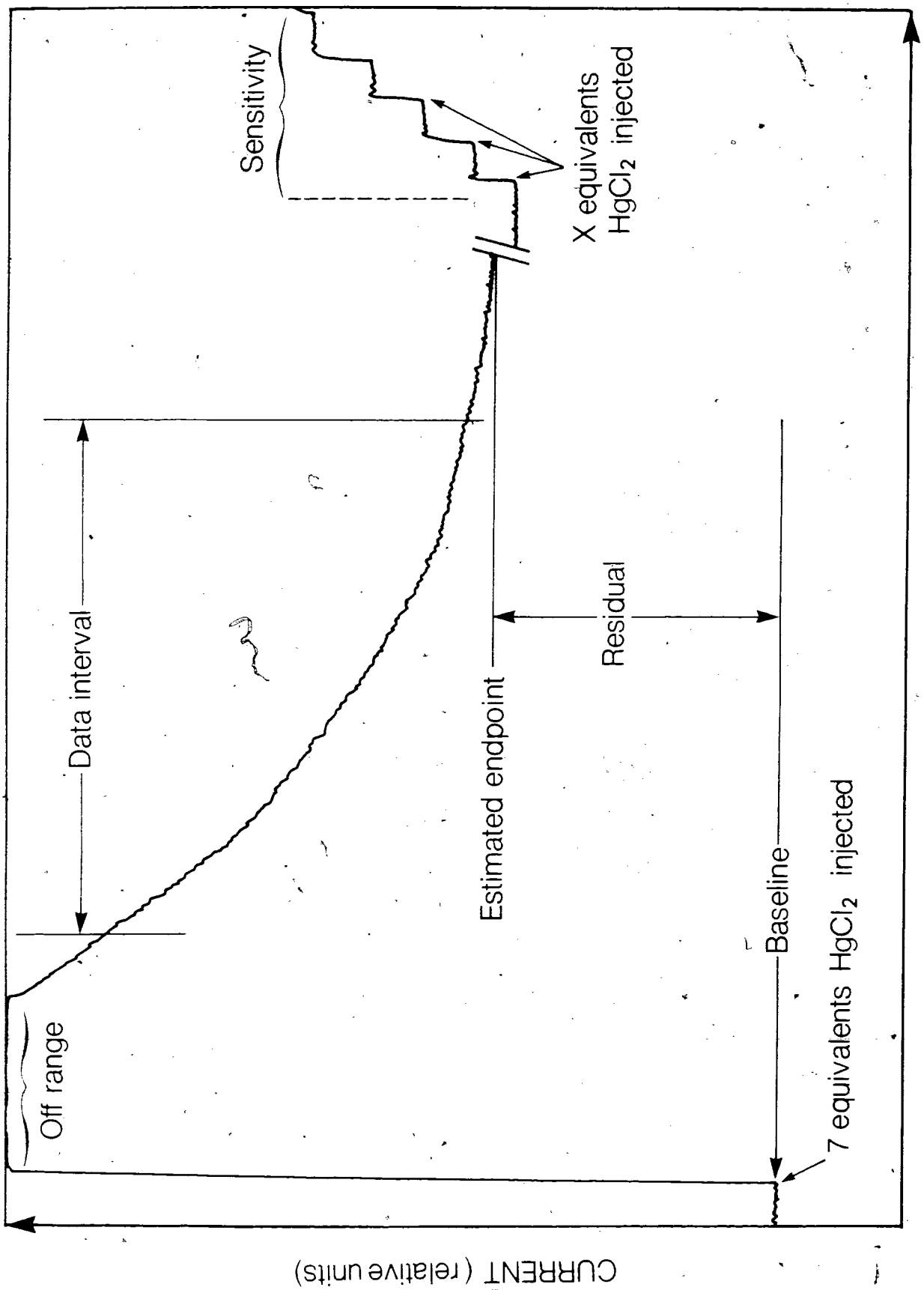

Sensitivity
X equivalents
$HgCl_2$ injected
Data interval
Residual
Estimated endpoint
Baseline
Off range
7 equivalents $HgCl_2$ injected
TIME (secs)
CURRENT (relative units)

Fig. 5. APL program ∇KINK used for second order analysis of hemoglobin titration results.

```
∇ KINK
 [1] 'COMMENT'
 [2] D←⍞
 [3] 'TYPE 5555 AT END OF DATA'
 [4] TX←⍳R←0
 [5] TX
 [6] 'DATA'
 [7] X←⍞
 [8] →(X=5555)/11
 [9] TX←TX,X
 [10] →7
 [11] 'ESTIMATED ENDPOINT E'
 [12] E←⍞
 [13] 'BASELINE'
 [14] B←⍞
 [15] W←0
 [15.1] H←E-B
 [16] KT←KA←⍳0
 [17] W←W+1
 [18] →((W+10)>⍴TX)/27
 [19] XA←TX[W]-E
 [20] XB←TX[W+10]-E
 [21] →(XB≤.00001)/27
 [22] →(H=0)/25
 [23] KT←KT,(⍟(XA+H)×XB÷(XB+H)×XA)÷(-H).
 [24] →17
 [25] KT←KT,(XA-XB)÷(XB×XA)
 [26] →17
 [27] ST←+/N×N←KT-KA←KA,(+/KT)÷⍴KT
 [28] W×⍳0
 [29] 'ENDPOINT'
 [30] E
 [31] 'K AVERAGE'
 [32] KA
 [33] 'DEVIATION'
 [34] DEV←(ST÷⍴KT)*.5
 [35] DEV
 [36] 'INDIVIDUAL K VALUES'
 [37] KT
 [38] F←((+/KT[⍳5])+(-(+/KT))+(+/KT[⍳((-5)+⍴KT)]))×DEV×40÷KA
 [39] 'NEXT ITERATIVE STEP'
 [40] F
 [41] E←E+F
 [42] R←R+1
 [43] →((DEV÷KA)>.01)×(R<10)×((|F)>.01)/15
 [44] 'OUT'
 [45] →0
∇
```

this would be the interval after the first 1/2 equivalent had reacted and before the last 1/10 equivalent would react. A minimum of twenty discrete data points would be inserted into the program along with the baseline and the endpoint which was estimated from the measured sensitivity and the calculated excess $HgCl_2$ which should remain in solution. (Most experiments were conducted with a 1 equivalent excess of $HgCl_2$ in solution.)

The program would then select successive data sets separated by 10 time intervals and fit them to an analytic description for a second order reaction of the form

$$kt = \frac{1}{a-b} \ln \frac{b(a-x)}{a(b-x)}$$

where a and b represent the initial concentrations of the reactants. As previously stated, the starting time for the reaction was ambiguous so time (t) and initial concentrations were not known. However, a-b would be equivalent to the residual (excess) in the reaction and if the reaction endpoint were also known then the rate equation could be rewritten as:

$$kt = \frac{1}{-Res} \ln \frac{(X_o + Res-e)(X_1-e)}{(X_1 + Res-E)(X_o-e)}$$

where t = time interval between X_o and X_1

Res = residual; $HgCl_2$-Hb

X_o, X_1 = two successive points along the curve

e = reaction endpoint

Given an estimated endpoint the program would compute at least ten separate kt values. These values would tend to diverge if the value of e was incorrect. By a completely arbitrary procedure the program would evaluate the divergence and estimate a better endpoint. The procedure would be repeated using the new e until the divergence of the kt values was negligible, specifically until the standard deviation for the computed kt values was less than 1% of the mean.

Once the data had been fitted to a second order decay curve the theoretically extrapolated reaction endpoint was compared with the endpoint expected from the respective concentrations of reactants. Any major discrepancies resulted in the rejection of the data.

I. STOICHIOMETRY OF THE Hb + Hg^{+2} REACTION

INTRODUCTION

Amino acid sequence analysis of human hemoglobin (11) has shown that there are 2 cysteine groups in each β chain and one in each α chain making up a total of six per tetramer. The β93 cysteine has been identified as the "reactive" group (10,22,25) in that it reacts within milliseconds with sulphydryl reagents such as heavy metal ions, phenylmercury compounds etc. The four remaining -SH groups at α104, β112, traditionally the "masked" or "unreactive" -SH groups were long considered to be unreactive with -SH reagent except under denaturing conditions (12,24). In recent years (1967-74) there have been an increasing number of reports on the slow reaction (a time scale of hours or days) of these groups with phenylmercury compounds under neutral conditions (26-29).

Early work on the titration of the -SH groups of hemoglobin (30-33) indicated that between 2 and 4 -SH groups of Hb A and Hb E were readily reactive and that another four would react on denaturation of the protein.

By 1962 the -SH titre of Hb A had been settled at 6 -SH per Hb tetramer with the first 2.2 molar equivalents being characterized as "reactive" and the remaining 3.8 equivalents as "unreactive" (34,35). A comprehensive review was published by R. Cecil (12) in 1963; the general conclusions which he formulated at that time remain the most recent published statement with respect to the mercurimetric

titration of hemoglobin -SH groups. His conclusions were:

i) In addition to reacting with the "reactive" SH groups, sulphydryl reagents such as mercuric chloride and phenylmercuric hydroxide would react with the "unreactive" -SH groups in slightly denaturing conditions.

ii) One pair of the four "unreactive" groups was more reactive than the other pair.

iii) The reaction of the unreactive groups was sensitive to the ligand state of the Hb molecule.

Though Cecil published two further papers (36,37) on the possible function of the unreactive -SH groups of Hb A, the apparent usefulness of -SH titration was eclipsed by amino acid sequence analysis and X-ray diffraction techniques. The reaction of the masked -SH groups of Hb A with PMB resurfaced in 1967 as a technique for separating hemoglobin subunits (29). Since that time the kinetics of the Hb cum PMB reaction have received some attention (26,38). Kinetic investigations will be discussed in detail in the next section; there are 4 further publications which have some indirect bearing on the stoichiometry of the Hb -SH reagent reaction.

Enoki & Tomita (39) reacted various hemoglobin types with $HgCl_2$ in neutral solution for 4 hours at 0^oC and were able to demonstrate the formation of hybrids, indicating reversible monomer formation. Five equivalents or more of Hg^{+2} per tetramer had to

be reacted in order to produce monomers.

Bucci (27) showed that PMB reacts eventually with all six -SH groups of native Hb A in neutral solution. Rosemeyer & Huehns (29) found that the reaction of PMB with the -SH groups of Hb A was salt dependent, reaching a maximum at .1 - .2M NaCl at pH 6. Fetal hemoglobin (Hb F) which does not have a cysteine residue at position β112 did not react beyond the β93-SH group and did not dissociate. Complete dissociation was found only with Hb's having two -SH groups on the β chain. They concluded that -SHβ112 reacts relatively rapidly in competition with -SHβ93; that -SHα104 is the last to react and would not react at all in the absence of a previously reacted β112 group.

This last finding was refuted by Ioppolo (40) in 1969. He found that Hb F would react with PMB and subsequently dissociate but the time course, optimal pH and ionic strength for the reaction differed from Hb A.

Hindsight is of great advantage in interpreting experimental literature; initially it was not at all clear whether 2,6 or more equivalents of Hg^{+2} would react with Hb A. The state of the art at the initiation of this study was epitomized by the following statement from Cecil (12).

> "Although the unreactive -SH groups can be titrated only after the hemoglobins have been denatured, they will react slowly with heavy metal reagents in the absence of denaturing agents."

RESULTS AND DISCUSSION

The "Unreactive" -SH Groups

Titrations of Hb A (Human hemoglobin) with $HgCl_2$ under conditions specified by other investigators (18) indicated that at least 2 equivalents of Hg^{+2} per Hb tetramer were reacting. The titration endpoint was poorly defined due to a slow but steady downward drift of the current beyond the 2 equivalent point (B in Fig. 6).

If the current reading was not taken until the observed drift became negligible, i.e. no observable deflection of the galvanometer over a 20 to 30 second period, the reaction endpoint resolved itself at 4 equivalents (C in Fig. 6). This was a particularly significant result in that it indicated that at least one of the "unreactive" -SH groups was reacting at a finite rate.

When the effects of various concentrations of halide salts in the titration medium were investigated (Figs. 7,8), it became evident that all of the "unreactive" -SH groups were reacting with $HgCl_2$ but under some circumstances at rates too low to be observed with the apparatus being used. It became apparent why Stricks and Kolthoff (18) and Benesch et al (31) had been unable to observe titration endpoints in low ionic strength media; such a titration illustrated in Fig. 8 is apparently without a well defined endpoint. This behaviour had been interpreted as indicating an indefinite stoichiometry for the Hg^{+2} -Hb reaction in low salt media, whereas apparently it results from the reaction of the "unreactive" -SH groups.

Fig. 6. Typical mercurimetric titration curves for hemoglobin at 50 μM. heme in 500mM KCl, 100mM Phosphate, pH 7.6. Curve A was obtained in the absence of Hb. Curve B is a rapid titration of Hb with $HgCl_2$ with 20 second intervals between successive mercury aliquots. Curve C represents a slow titration with 2 min. intervals between successive aliquots. The arrows indicate a perceptible diminution of the current during the 2 min. interval.

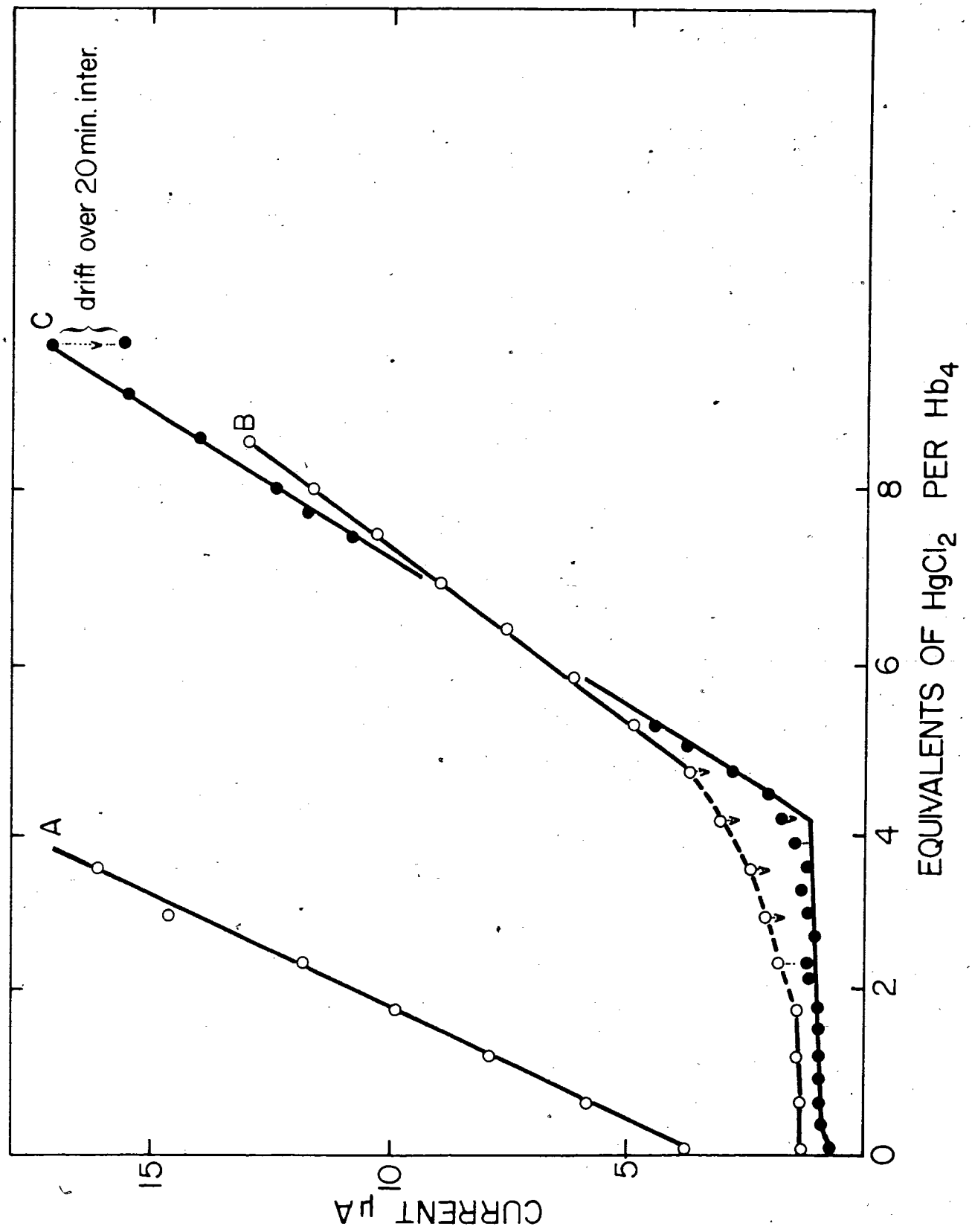

drift over 20 min. inter.
C
B
A
CURRENT μA
15
10
5
0
2
4
6
8
EQUIVALENTS OF $HgCl_2$ PER Hb_4

Fig. 7. Mercurimetric titrations of HbA at 50 μM. heme in 100mM phosphate, pH 7.6 plus various concentrations of NaBr. The time interval between successive aliquots of mercury was 2 min.

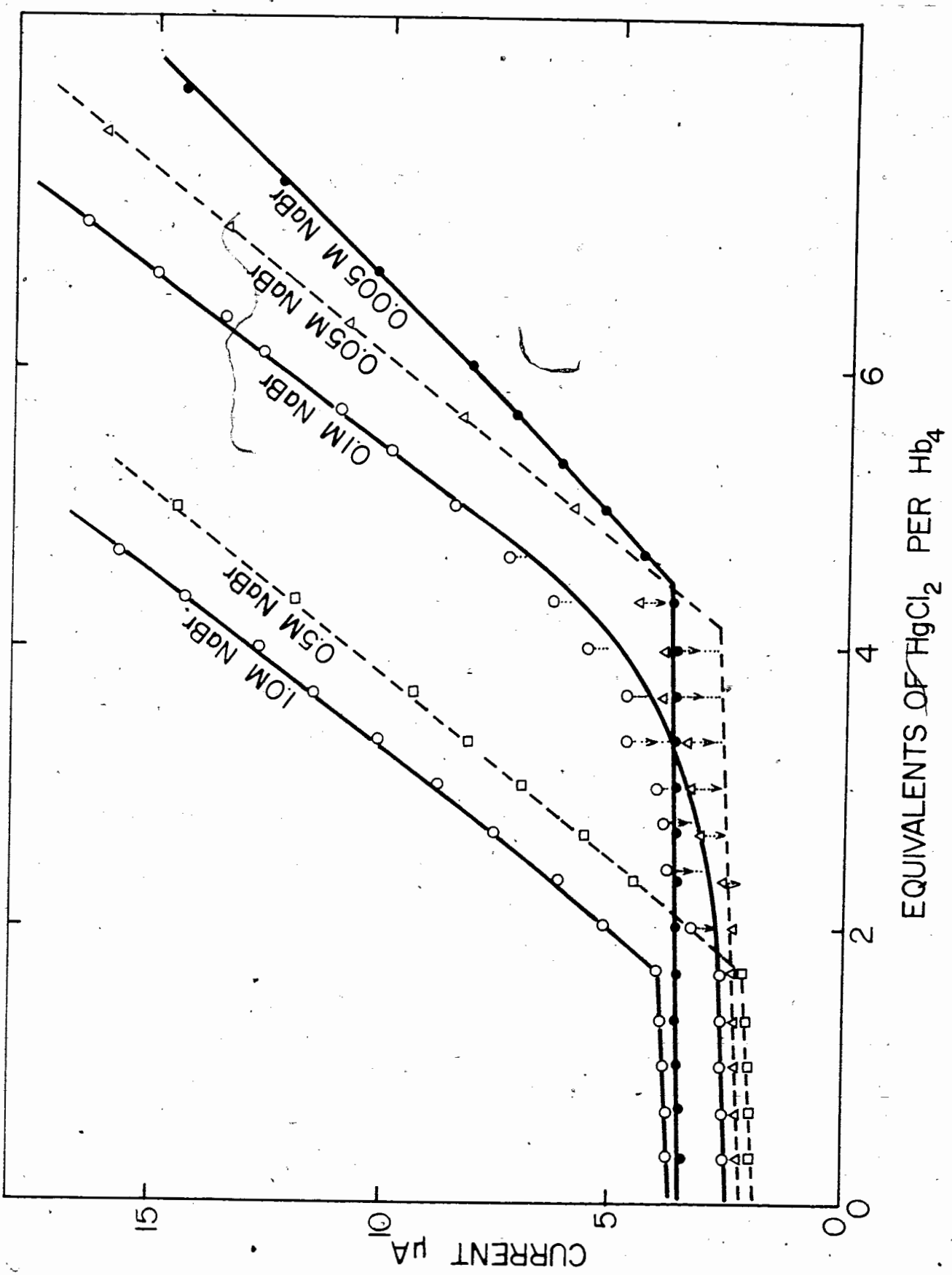
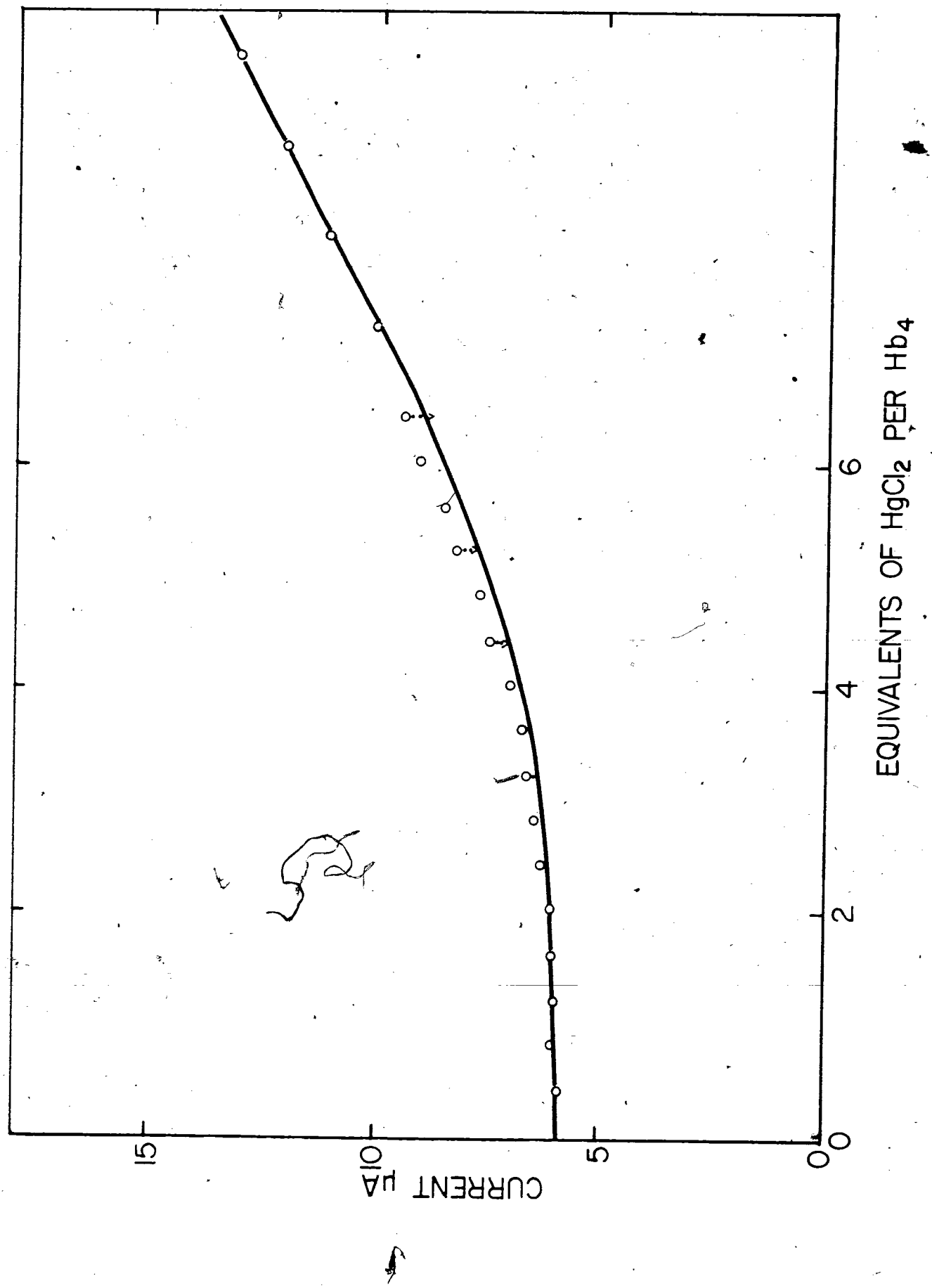

1.0M NaBr
0.5M NaBr
0.1M NaBr
0.05M NaBr
0.005M NaBr
CURRENT μA
0
5
10
15
0
2
4
6
EQUIVALENTS OF $HgCl_2$ PER Hb_4

Fig. 8. Mercurimetric titration of Hb A at 50 μM. heme in 100 mM phosphate, pH 7.6 plus 160mM NaCl. Time interval between aliquots: 1 min. There is no well defined reaction endpoint.


CURRENT μA
15
10
5
0
0
2
4
6
EQUIVALENTS OF $HgCl_2$ PER Hb_4

Several experiments were conducted to rule out any other interpretation of these results. The consumption of mercuric ion by the amperometric apparatus was found to be an order of magnitude too small to account for any of the observations. The possibility of reactions with impurities or with denatured protein was ruled out by the following observations:

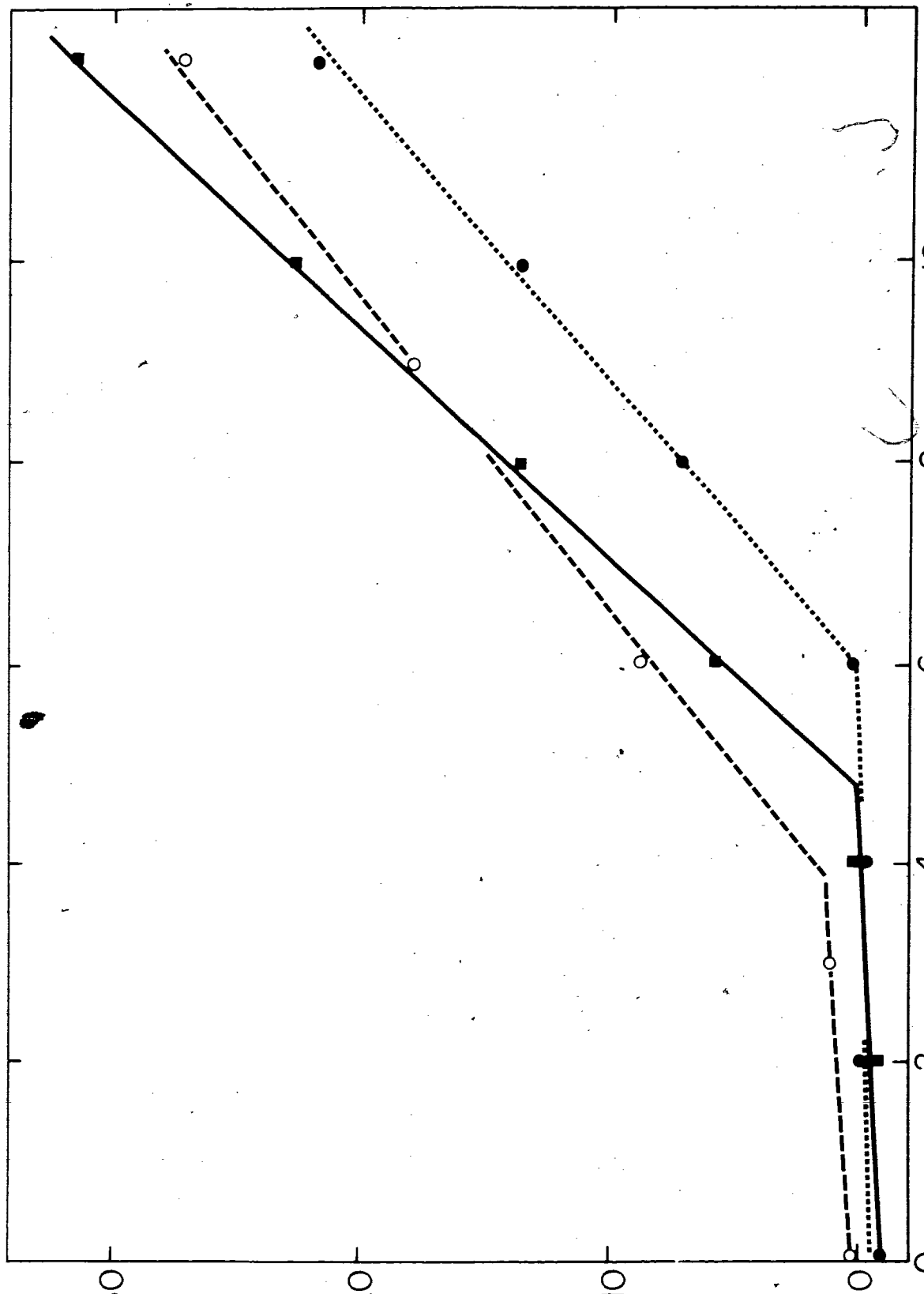
a) Hb A was heat denatured at 45°C in the presence of an excess of $HgCl_2$ (in 100mM Phosphate, 500mM KCL). Under these circumstances six and only six equivalents of mercury reacted. With Equine hemoglobin (Hb E) and with "Cord Blood" (60% Hb F + 40% Hb A) the reaction stoichiometry similarly corresponded to the number of -SH groups in the particular Hb sample.

b) Hb A, Hb E and "Cord Blood" hemolysate were incubated with various amounts of $HgCl_2$ for 48 hours while taking intermittent readings of Hg^{+2} concentration (Fig. 9). The stoichiometry of the reaction of Hg^{+2} with the -SH groups was 1:1 with no further significant binding of Hg^{+2} with denatured protein or impurities.

During the investigation of reaction kinetics to be described in the next section the reaction stoichiometry was observed to be 6 equivalents $Hg^{+2}/Hb_4 \pm 5\%$ for several hundred titrations under a wide range of conditions.

Fig. 9. The stoichiometry of the reaction of HbA, HbE and "Cord Blood" hemolysate (~60% HbF + ~40% HbA). The hemoglobins were incubated in 100mM Phosphate, pH 7.6, 500mM KCl plus varying amounts of $HgCl_2$ for 48 hours at 20°C. The unreacted $HgCl_2$ was determined intermittently with the amperometric apparatus.

··· HbA, --- HbE, — Cord Blood


CURRENT μA
30
20
10
0
0
2
4
6
8
10
EQUIVALENTS OF $HgCl_2$ PER Hb_4

Weak binding of Hg^{+2}

In addition to the strong binding of Hg^{+2} at the six -SH groups of Hb A, two equivalents of Hg^{+2} were observed to be weakly bound at an unidentified locus.

The existence of this reaction was inferred from a sensitivity anomaly in the amperometric apparatus. After the complete reaction of 6 equivalents of Hg^{+2} with Hb A, small aliquots of $HgCl_2$ were added to the titration medium and the response of the apparatus recorded for each increment in $HgCl_2$ concentration.

It was evident from the plot of instantaneous sensitivity, $\frac{dI}{d(Hg^{+2})}$ shown in Fig. 10, that a reversible or concentration dependent reaction was taking place. If the instantaneous sensitivity was assumed to be constant at its limiting value then the quantity of Hg^{+2} being sequestered and the concomittant association constant could be obtained from an integrated plot of the type shown in Fig. 11. Assuming a single reaction, an association constant K_ζ was computed as $K_\zeta = 1.2 \times 10^4$ liter/M.heme as compared to $K \simeq 10^{21}$ for the binding of Hg^{+2} to cysteine (12).

A weak binding site would be expected to "saturate" at a higher molar excess of $HgCl_2$ per Hb tetramer as the hemoglobin concentration was reduced. The dashed line in Fig. 10 indicates this effect.

This interpretation of the sensitivity anomaly is corroborated by the finding that the apparent second order rate constant for the

Fig. 10. The instantaneous sensitivity of the amperometric apparatus to $HgCl_2$ in various concentrations of HbA plus 100mM NaCl, 10mM Phosphate, pH 7.6. For clarity the vertical displacement between the three curves is arbitrary, the actual value of the limiting current is indicated on the Figure. The dashed line indicates the increasing saturation of the weak binding site with increasing concentration.

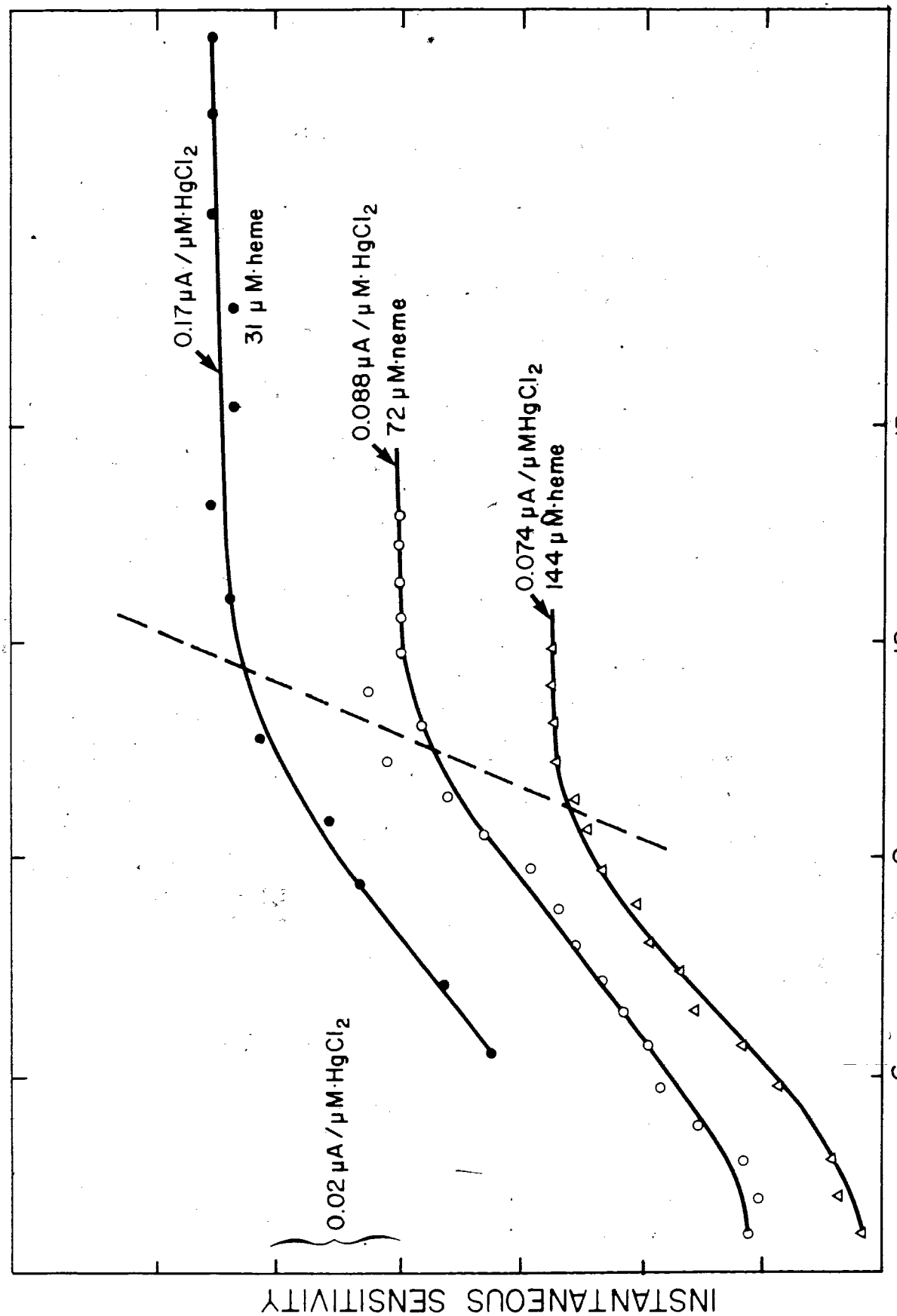
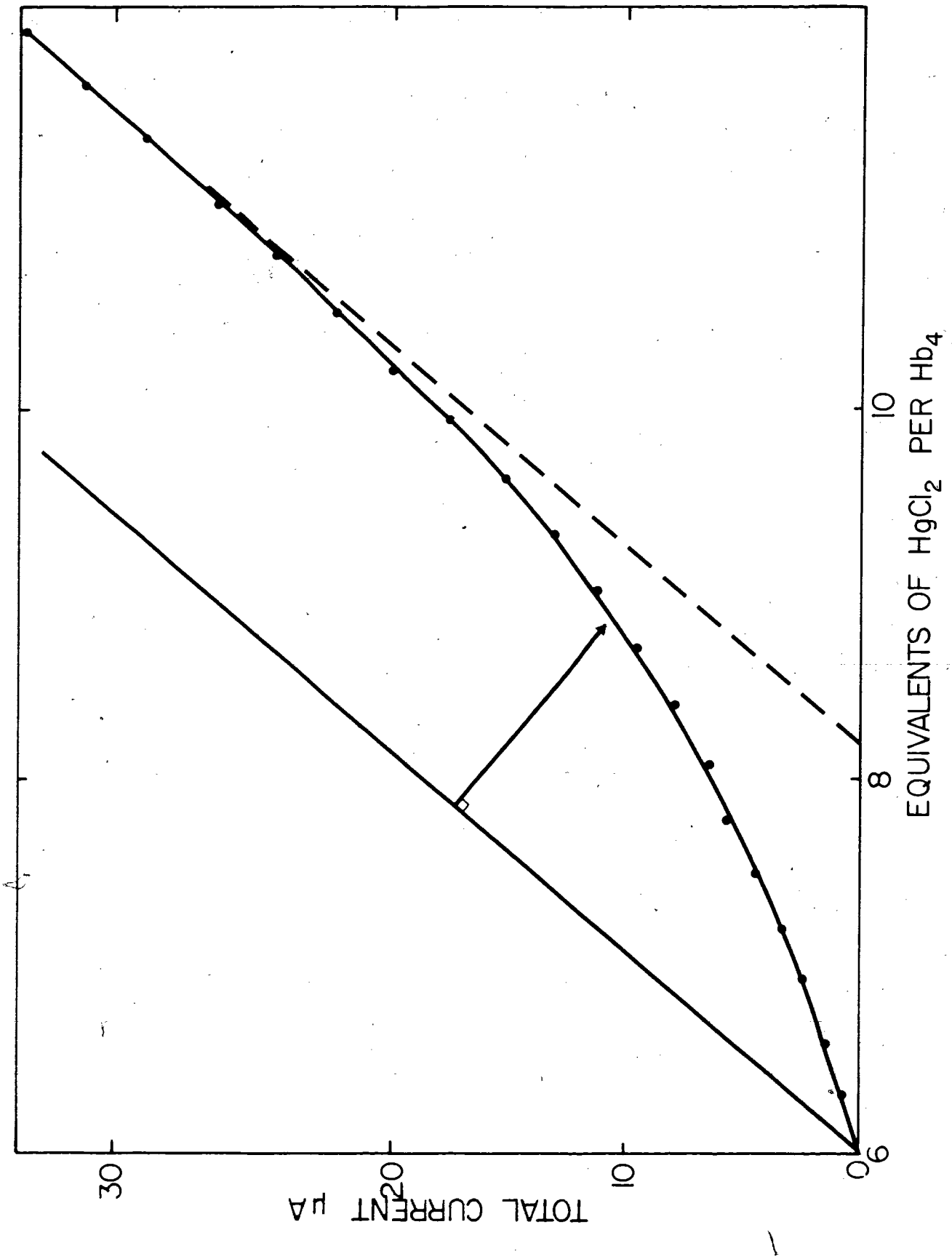
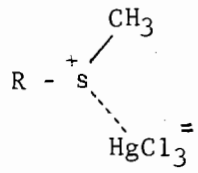

0.17 μA / μM·HgCl2
31 μ M·heme
0.088 μA / μ M· HgCl2
72 μ M·neme
0.074 μA / μ MHgCl2
144 μ M·heme
0.02 μA / μM·HgCl2
INSTANTANEOUS SENSITIVITY
6
9
12
15
EQUIVALENTS OF HgCl2 PER Hb4

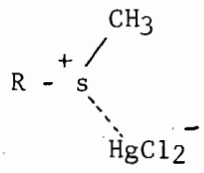
Fig. 11. The integral of the instantaneous sensitivity curve for 144 μM. heme represented in Fig. 9. The stoichiometry of the reaction was approximated by extrapolating the terminal slope of the curve. The association constant was computed from the differences indicated by the perpendicular.


TOTAL CURRENT μA
30
20
10
0
6
8
10
EQUIVALENTS OF $HgCl_2$ PER Hb_4

reaction of the unreactive -SH groups with $HgCl_2$ tends to increase with increasing $HgCl_2$ concentration as shown in Fig. 12. Chiancone (26) observed a similar effect for the reaction of PMB with the "unreactive" -SH groups and suggested that some association equilibrium might be involved.

As the reversibly bound $HgCl_2$ amounts to 2 equivalents per tetramer it should be possible to associate it with a particular site on the α or β subunit. There is a methionine group located at α76 in the bend between the E and F segments; possibly this group is the reactive group as it is the only accessible Met in the tetramer. Met α32 is involved in the heme contact and Met β55 in the α,β, interface (41). Therefore they are not likely to be readily accessible. Thio ethers readily undergo nucleophilic attack by mercury salts (12) and though this reaction has not been observed to occur with methionine, the existence of compounds





would not be unlikely. The heme iron of cytochrome C is complexed to a Met residue in exactly this way (42). No other amino acid groups in Hb A seem to be as likely candidates for the locus of this particular reaction. Though such a reaction might occur with imadazole groups, their numbers and location on the Hb molecule are inappropriate.

II. KINETICS OF THE REACTIONS OF $HgCl_2$ WITH THE UNREACTIVE -SH GROUPS OF HEMOGLOBIN

Introduction

Structural analysis of Hb A (43-45) has shown that the β93 cysteine is located on the exterior surface of the Hb tetramer next to the "proximal" histidine which is the major point of attachment for the heme iron. This close association has been exploited in studies involving interactions between the ligand state of the heme group and the reactivity of -SHβ93 and vice versa (5,21-24,46-50).

The α104 and β112 groups are located in the $\alpha_1\beta_1$ interface of the hemoglobin tetramer (41,43,44,51). As this interface is not normally exposed to solution (51) the relatively low reactivity of these -SH groups might be explained. If the low reactivity does, in fact, reflect the sheltered condition of these cysteines it would be expected that conditions which induce dimerization or monomerization of the hemoglobin tetramer would also affect the rates of reaction of the "masked" -SH groups (26,36,37). By extension, since the dimer-tetramer equilibrium is related to the ligand state of the molecule (21,52-54) the relative reaction rates might also be related to the ligand state.

The possibility of using -SH groups in the $\alpha_1\beta_1$ interface as probes of subunit interactions has been investigated by several workers.

Cecil & Snow (34,35) were the first to recognize the possibility even though at that time, 1962, the structure of hemoglobin was largely unknown. They determined that the relative reactivities of various hemoglobins fell into the order Met Hb > deoxy Hb > CO Hb. The kinetics of the sulphydryl reactions were thought to be indeterminate as it was believed that a single -SH group would bind more than one Hg^{+2} ion or phenylmercury molecule. Oxyhemoglobin could not be investigated due to the interference of oxygen with their mercurimetric technique.

The separation of hemoglobin subunits by complete reaction with PMB was initiated by Bucci et al (27,28). Though they were not interested in ligand effects per se, they were aware that the yield of separated α & β chains was sensitive to the ligand state of the Hb (22,49).

Rosemeyer & Huehns (29) explored the parameters controlling the reaction of PMB with the unreactive -SH groups in order to optimize the yield of separated chains. Though reaction rates were only inferred from electrophoretic yield they reached the following conclusions:

a) The reaction of O_2Hb with excess PCMB increases with the NaCl concentration reaching a maximum at about 0.2M NaCl, pH 6.0.

b) Sulphydryl groups β93, β112 react rapidly whereas -SHα104 is slow to react.

Finally in 1970 Chiancone et al (26) published a study on the

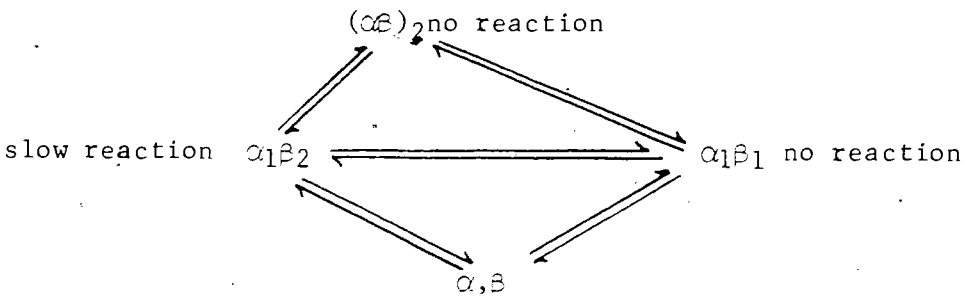
kinetics of the reaction between PMB and the -SH groups of Hb A. Reaction rates were measured using the spectrophotometric technique of Boyer (55). The effects of pH, ionic strength and protein concentration were observed for COHb, & O_2Hb.

a) Reaction rates tended to increase with decreasing pH and were sensitive to the nature of the buffer system.

b) The reaction rate reached a maximum at 0.1M NaCl dropping only slightly over the range from 0.1 - 1.0M NaCl.

c) The apparent rate decreased with increasing hemoglobin concentration and increased with increasing PMB concentration.

They tentatively suggested the reaction scheme illustrated below:



The existence of a recent publication on the reactivity of -SH groups of chicken Hb should be mentioned. Cirotti & Geraci (38) found that Hb1 with eight -SH groups bound six equivalents of PCMB in the oxy form and only four equivalents in the deoxy form; Hb2, with 10 -SH groups bound eight equivalents of PCMB regardless of ligand state.

As demonstrated in Part I the unreactive -SH groups of Hb A,E,F, are all reactive with $HgCl_2$ on a time scale compatible with the response time of the amperometric apparatus described in Methods. In order to ascertain the feasibility of a probe for subunit interactions a number of preliminary experiments was conducted in order to determine whether or not the reactions being observed were sensitive to the ligand state of Hb. The results were encouraging. With Hb A, Rate II^2 was more or less independent of ligand state (Fig. 18). On the other hand Rate III^2 was appreciably affected by the ligand state of Hb A (Fig. 21).

On the basis of this observation most of the ensuing experimental work was done in respect to Rate III of Hb A, though Rate II was characterized to some extent. Rate III was convenient for experimental purposes in that the reaction went to completion on a time-scale of minutes in a titration medium such as Buffer Ⓟ .

THEORETICAL

In formulating a comprehensive theoretical rate expression for the reaction of $HgCl_2$ with hemoglobin in a titration medium containing Cl^- ions three factors had to be taken into account.

i) Which species of Hb is reactive: monomer, dimer or tetramer?

ii) The dissociation constant for $HgCl_2$ in distilled water is rather low; in aqueous solution with any appreciable concentration of Cl^- the mercuric ion will appear

primarily in the forms $HgCl_4$, $HgCl_3^-$ and $HgCl_2$ (56,57). The two former species, though more abundant than $HgCl_2$, are not as likely to be reactive with R-SH or $R-S^-$ because of their net negative charge. Whatever the case, a determination of an apparent rate constant would have to account for the actual concentration of the reactive species of mercury:

iii) The weak binding of 2 equivalents of $HgCl_2$ at a non -SH locus must be accounted for in any kinetic analysis.

The first two considerations can be dealt with rather simply as the factor in question becomes a constant which can be removed from the integral. For the hypothetical case where dimer is the reactive species of Hb in the solution, the rate equation can be written in standard form as

$$\frac{dx}{dt} = k(HGT-x)(D-y)$$

Assuming that the dimer - tetramer equilibrium remains unaffected by the reaction of dimer with Hg^{+2} and also that the dimer - tetramer turnover rate is rapid by comparison with the rate of the reaction; the value of y will then be proportional to the value of x.

$$y = x \frac{D}{HBT} \qquad (2)$$

The first assumption is probably not valid for the reaction system being studied but is a useful approximation. The second assumption is justified; Uzgiris and Goliversuch (58) have

recently measured tetramer-dimer dissociation constants for HbA. K_f the dissociation rate constant, was evaluated as $K_f = 9 \pm 8$ sec^{-1} for HbA in 0.1M NaCl.

Substituting (2) into (1)

$$\frac{dx}{dt} = K(HGT-x)(D-x\frac{D}{HBT}) \qquad (3)$$

multiplying the entire expression by

$$\Phi = \frac{HBT}{D}$$

one obtains

$$\Phi\frac{dx}{dt} = K(HGT-x)(HBT-x) \qquad (4)$$

the integrated rate equation will be the familiar 2nd order expression with an added constant:

$$K = \frac{\Phi}{t}\frac{1}{HGT-HBT}\ln\frac{HBT(HGT-x)}{HGT(HBT-x)} \qquad (5)$$

Thus, in the case that either monomeric or dimeric Hb is the reactive species the apparent rate constant will be seen to vary as Φ i.e., as either

$$\frac{HBT}{D} \text{ or as } \frac{HBT}{M}$$

The question of identifying the reacting species of $HgCl_x$ can be handled in an identical fashion. The exchange rate for chloride on Hg is in the order of 10^{-7} - 10^{-10} seconds (73,74) so the assumptions made in respect to Hb dimer - tetramer turnover are valid for the various species of $HgCl_x$. The apparent rate constant will be seen to vary as $\ominus$ where

$$\Theta = \frac{Hg\ Total}{HgCl_x} \qquad (6)$$

To account for the weak binding of $HgCl_2$

$$K_{\xi} = \frac{HgHb}{(Hg)(Hb)} \qquad (7)$$

But Hb can be expressed as Hb = HBT - HgHb; substitute this expression into (7) and solve for HgHb

$$HgHb = K_{\xi} \frac{(Hg)(Hb)}{1 + K_{\xi}\ Hg} \qquad (8)$$

The instantaneous Hg concentration in a solution in which -SH groups are reacting will then be:

$$Hg = HGT - x - HgHb \qquad (9)$$

and the rate equation will be:

$$\frac{d\ Hg}{dt} = -k\ (Hg)(HBT - x) \qquad (10)$$

Substituting (8) & (9) into (10)

$$\frac{d\ Hg}{dt} = -k\ (Hg)\ (HBT - HGT + Hg + \frac{K_{\xi}\ Hg\ HBT}{1 + K_{\xi}\ Hg}) \qquad (11)$$

By the binomial expansion

$(1 + K_{\xi}\ Hg)^{-1}$ will become

$$(1 + K_{\xi}\ Hg + K_{\xi}^{2}Hg^{2} - K_{\xi}^{3}Hg^{3} \ldots) \qquad (12)$$

Taking the first two terms of (12) as a first approximation and substituting into (11):

$$\frac{dHg}{dt} = k\ (Hg)\ (a + Hg\ (1 + K_{\xi}\ HBT - K_{\xi}^{2}\ HBT\ Hg)) \qquad (13)$$

where $a = (HBT - HGT)$.

Equation (13) can be written in the form

$$\frac{dx}{dt} = k\ (x)\ (a + bx + cx^2) \qquad (14)$$

where $x = Hg$

$a = (HBT - HGT)$

$b = (K_{\zeta} HBT + 1)$

$c = (- K_{\zeta}^{\ 2} HBT)$

and integrated to give

$$kt = \frac{1}{2a} \ln \frac{x^2}{X} - \frac{b}{2a} \left(\frac{-2}{\sqrt{-q}} \tanh^{-1} \frac{2cx + b}{\sqrt{-q}} \right)$$

where

$q < 0$

or

$$\frac{2}{\sqrt{q}} \tan^{-1} \frac{2cx + b}{\sqrt{q}}$$

where

$q > 0$

$q = 4ac - b^2$

$X = a + bx + cx^2$

RESULTS AND DISCUSSION

Reaction Order

A primary consideration in the interpretation of reaction data was the assignment of reaction order. Though it was not essential to distinguish between a first order or second order reaction in order to correctly interpret ligand effects or temperature effects on the apparent rate constant (Fig. 21, Fig. 22), the effect of varying the Hb concentration can only be interpreted if a unique reaction

order is assigned. This point is best illustrated by the apparent concentration dependence shown in Fig. 24; a K_{app} proportional to $[Hb]^{-1}$ is precisely the relationship one would expect to observe if a first order reaction were being incorrectly analysed as second order reaction.

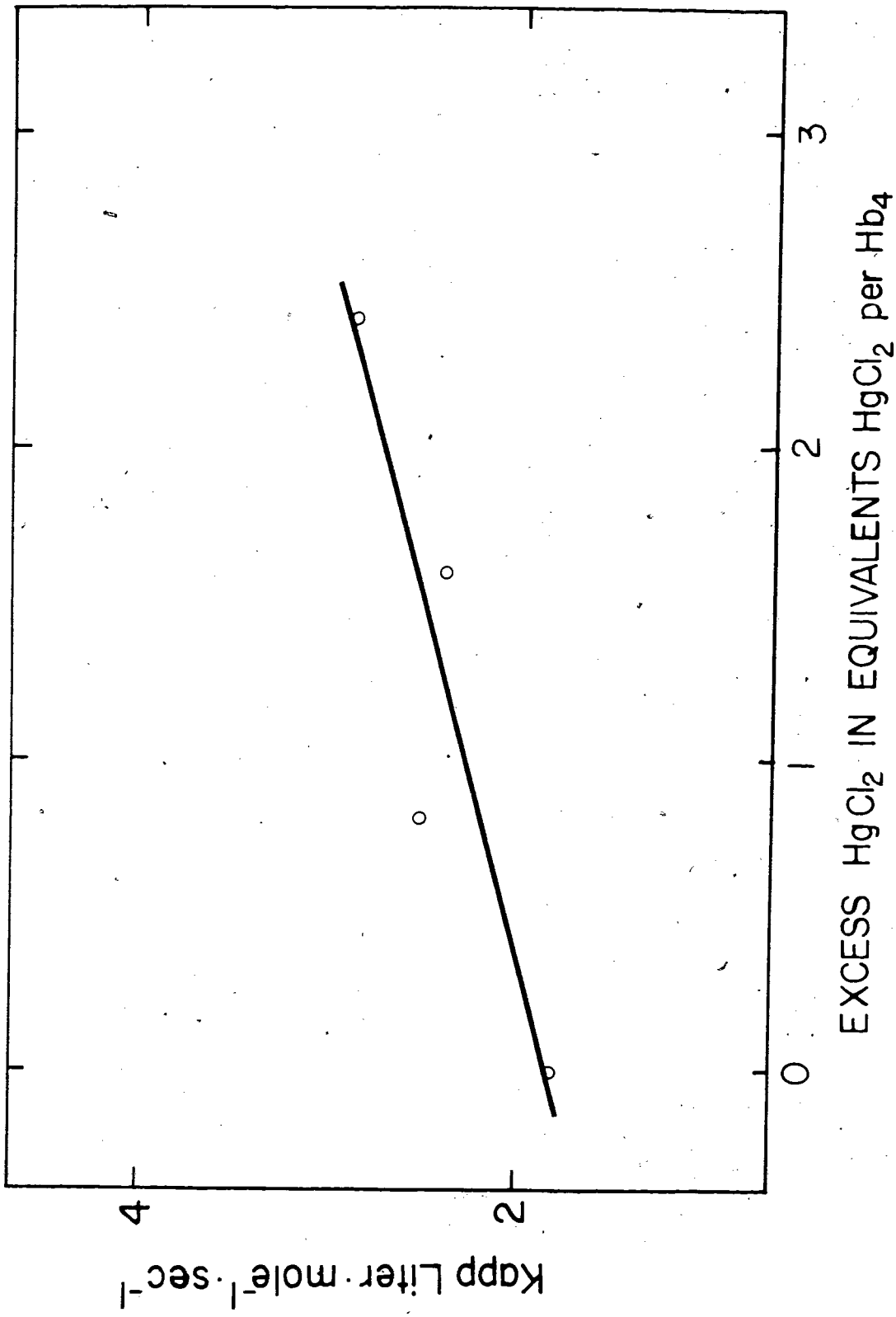
The method of curve fitting was unsatisfactory. Ambiguities as to the completion of the reaction were small but sufficient to preclude positive identification of the reaction as second order.

The overall reaction was determined to be second order by investigating the mercury concentration dependence of the apparent second order rate constant (Fig. 12). The rate constant is almost independent of $HgCl_2$ concentration increasing only slightly due to progressive saturation of the weak binding site. A consideration of the Hb concentration dependence (Fig. 26) would not lead to the conclusion that the overall reaction is second order. This discrepancy will be considered in later discussion.

Chiancone et al (26), working with the reaction of PMB with the unreactive -SH groups of Hb A, successfully treated the ensemble as a single pseudo first order reaction. As early as 1963 Cecil (12) had indicated that one of the two "unreactive" pairs was more reactive than the other. Rosemeyer and Huehns (29) also reached this conclusion.

The explanation for this discrepancy seems to lie in the difference between the reactions of PMB and $HgCl_2$ with Hb - SH groups. Although Ioppolo et al (40) did show that -SHα104 groups

Fig. 12. Variation of the apparent second order rate constant for Rate III as a function of $HgCl_2$ excess in 65 μM.heme, 100mM NaCl, 10mM Phosphate, pH 7.6.


Kapp Liter·mole^{-1}·sec^{-1}
4
2
0
1
2
3
EXCESS $HgCl_2$ IN EQUIVALENTS $HgCl_2$ per Hb_4

would react with PMB in the absence of an -SHβ112, the observed reaction half time was very long by comparison with -SHα104 reacting in sequence with a -SHβ112 group (26,29). This suggests, as concluded by Rosemeyer and Huehns (29) that the reaction of -SHβ112 with PMB facilitates the reaction of -SHα104 with PMB to the extent that these reactions can be considered to proceed in series rather than in parallel. Electrophoretic patterns of Chiancone (23) show that Hb A subunits are produced after the reaction of only 3 equivalents of PMB, supporting this conclusion. In such a situation the earlier phases of the reaction will be pseudo first order. In the experiments in which Chiancone (26) determined reaction order, his reactions were far from completion, due to the extremely slow reaction rate of PMB at low molar excesses.

This explanation is further corroborated by Chiancone's (26) finding that the activation energy for his reaction was 10 ± 2 Kcal/mole. This corresponds closely to the E_a reported here for Rate II, the reaction of -SHβ112 of Hb A (which would control the PMB reaction rate).

For the reaction of $HgCl_2$ with Hb A the facilitation of the reaction of SHα104 does not appear to occur, at least to the same extent. This is amply clear from the titration results Fig. 7 in which endpoints at 4 equivalents are clear cut under certain conditions.

Excepting where indicated, all of the apparent rate constants

have been computed using a standard second order rate expression. Equation (15) which introduces a correction for the weak binding group was applied to kinetic data randomly selected from the experimental results. Since the resulting correction factor amounted to only $\sim$ + 10%, which is much less than the scatter ($\pm$ 50%), the correction was considered to be negligible and was not applied.

SALT EFFECTS

The relationship between salt concentration and apparent reaction rate was investigated.

Figure 13 illustrates the effect of KCl concentration on reaction half-times. A tenfold increase in KCl concentration results in a decrease in half-times by a factor of 36. This result is almost identical to the effect of NaCl concentration (Fig. 14) which shows a 32 fold decrease in the apparent rate constant for the same concentration change.

As previously indicated mercuric ion will exist primarily as $HgCl_4^=$ and $HgCl_3^-$ in solution. The concentrations of various species of $HgCl_x$ as a function of Cl^- concentration have been computed using dissociation constants from Martell (32). As seen in Fig. 15 the ratio of $HgCl_2$ to Hg total drops by roughly 30 fold as Cl^- goes from 0.1M to 1.0M. None of the other species of $HgCl_x$ come close to matching the relationship observed in Figs. 13, 14.

It was assumed that $HgCl_2$ is the reactive species of mercury

Fig. 13. Reaction half-time for Rate III as a function of KCl concentration in the titration medium: 50 μM. heme, 100mM Phosphate, pH 7.6.

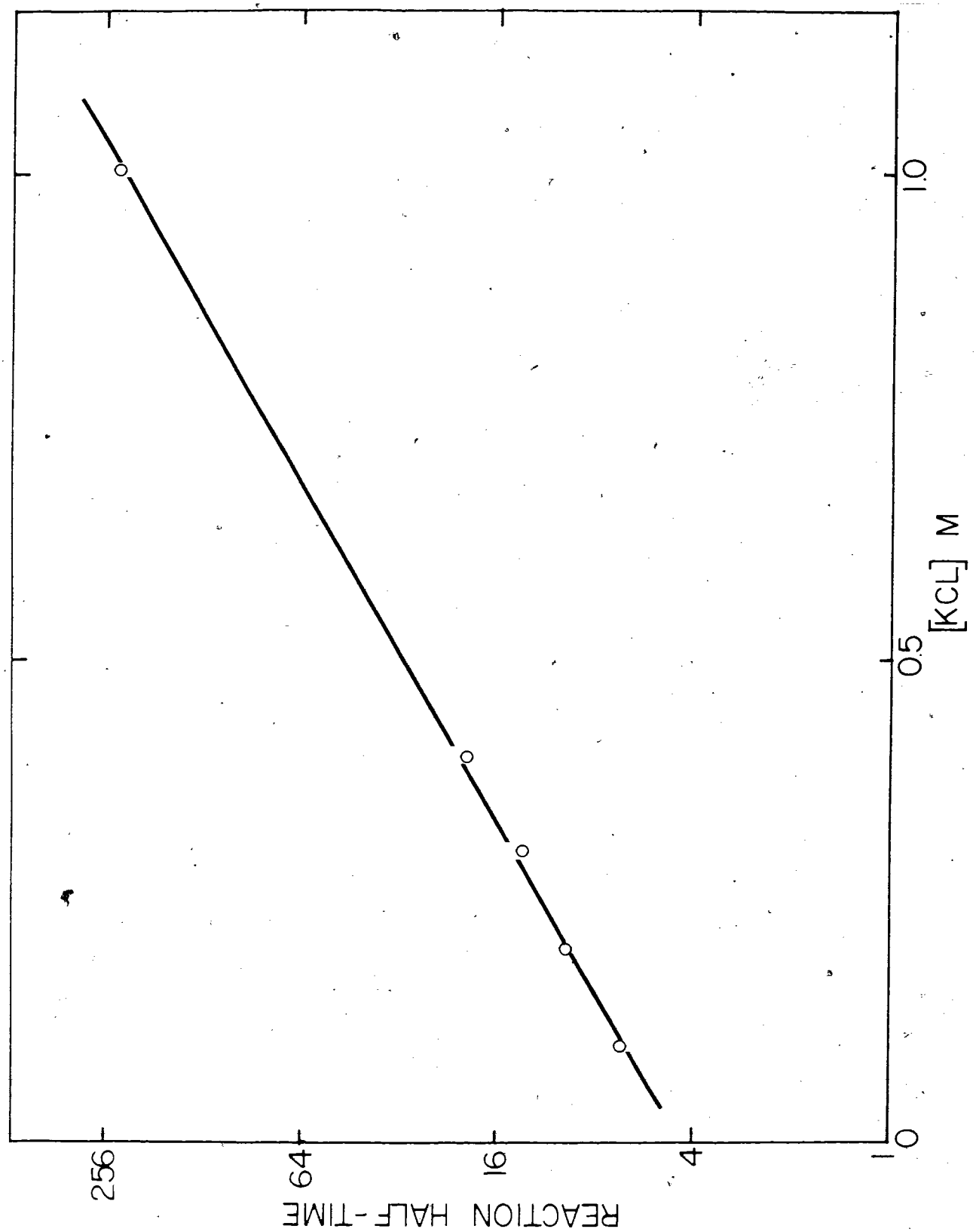

REACTION HALF-TIME
256
64
16
4
1
0
0.5
1.0
[KCL] M

Fig. 14. The effect of NaCl concentration on the apparent rate constant and on the Hb concentration dependence of Rate III in 10mM Phosphate, pH 7.6 at 21°C.

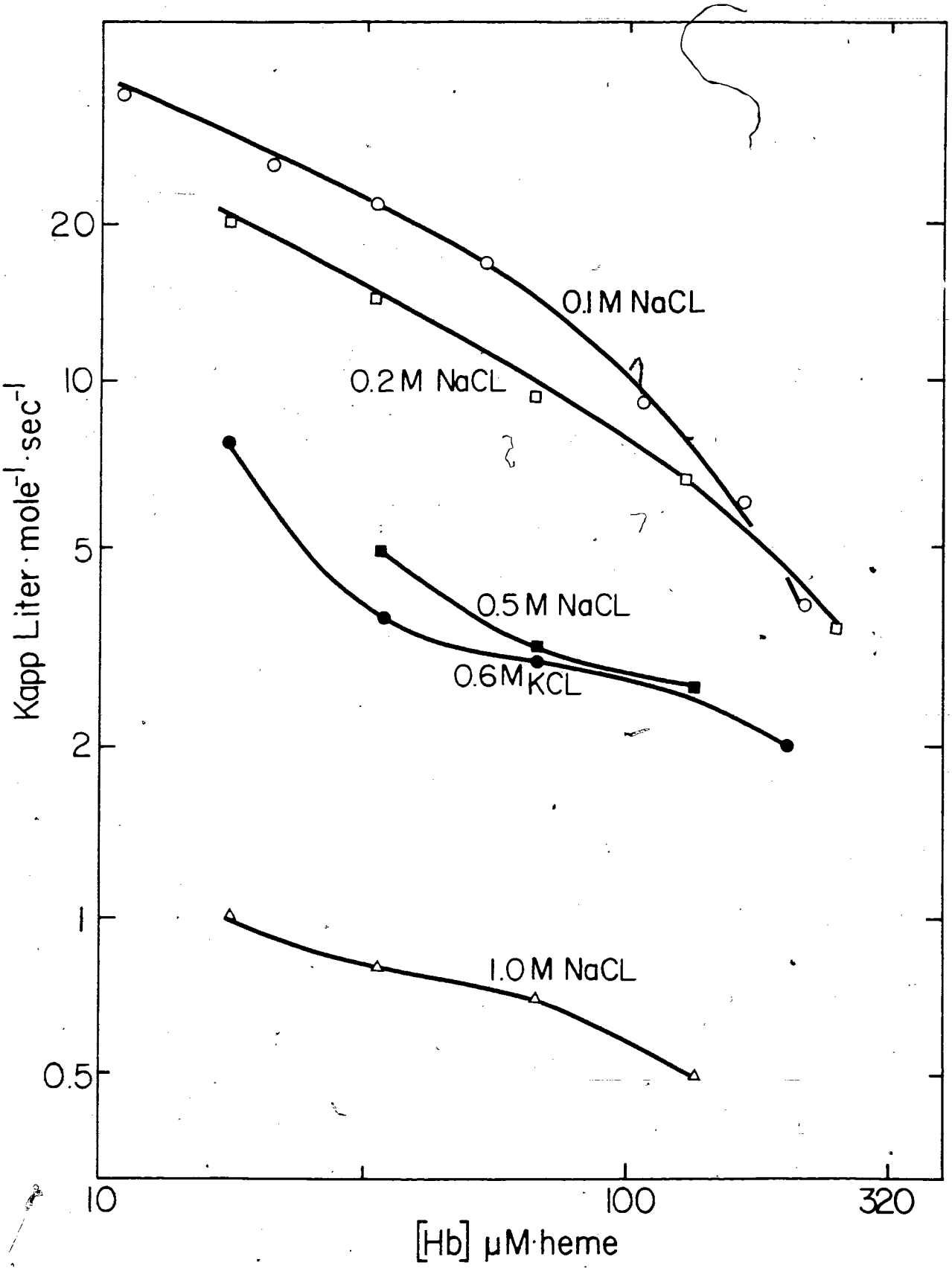
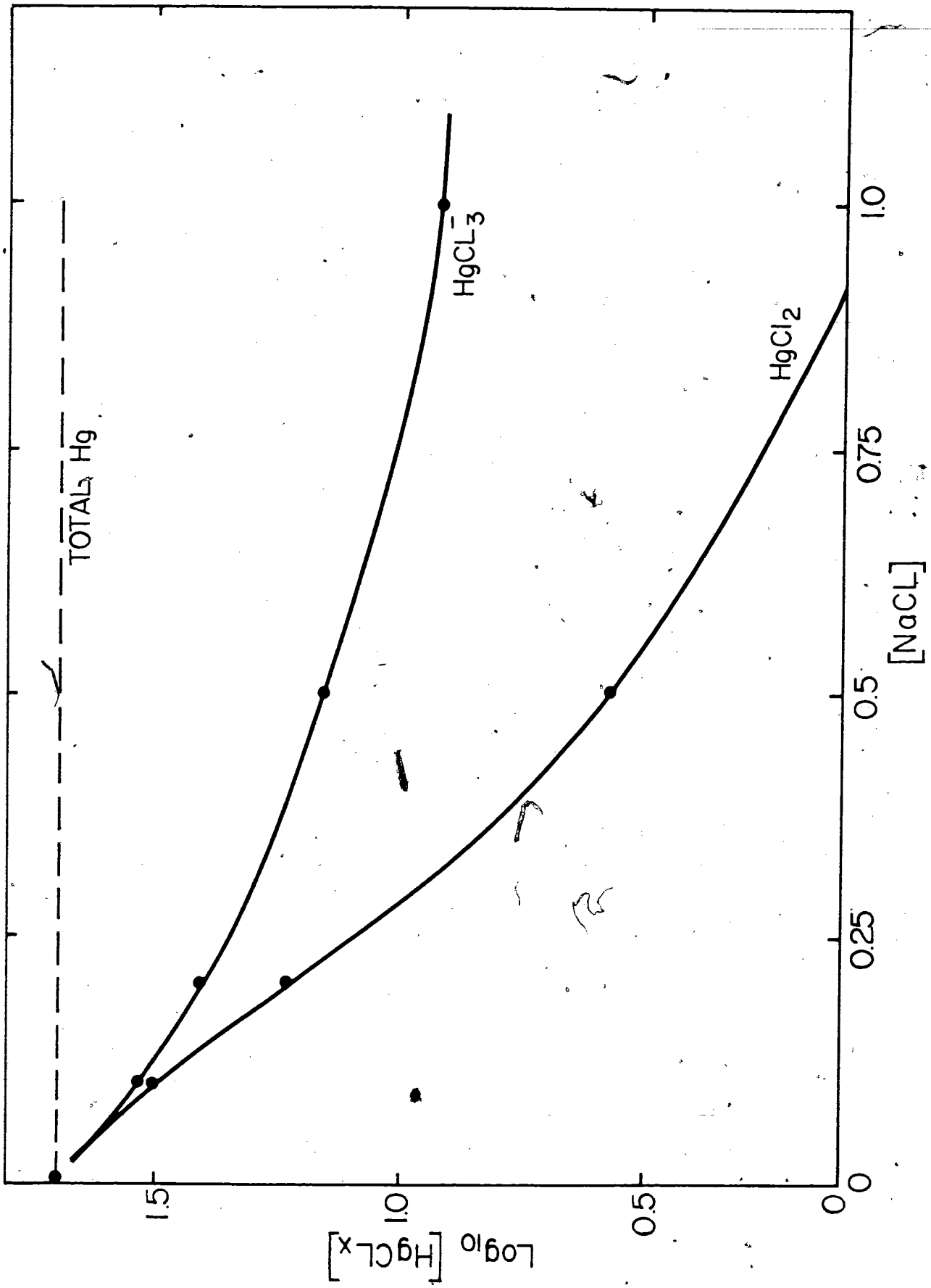

20
10
5
2
1
0.5
Kapp Liter·mole⁻¹·sec⁻¹
0.1 M NaCL
0.2 M NaCL
0.5 M NaCL
0.6 M KCL
1.0 M NaCL
10
100
320
[Hb] μM·heme

Fig. 15. The calculated concentration of various $HgCl_x$ species as a function of NaCl concentration. Constants used were: $\log_{10}K_4 = 0.69$ for $HgCl_3^-$, and $\log_{10}K_4K_3 = 1.68$ for $HgCl_2$ applied as indicated by Martell (32).


TOTAL Hg
$HgCL_3^-$
$HgCl_2$
Log_{10} [$HgCL_x$]
[NaCL]
1.5
1.0
0.5
0
0.25
0.5
0.75
1.0

and the value of Θ (see theoretical discussion) was calculated using as the dissociation constant log K_4K_3 = 1.68 (57). The apparent rates as shown in Fig. 14 were then corrected for "true" $HgCl_2$ concentration. The relationship between apparent rate and chloride concentration then appeared as in Fig. 16.

The relationship between the corrected K apparent and NaCl concentration coincides closely to the relationship which Chiancone (26) found for the corresponding PMB reaction.

The β112 Cysteine Group

Hb E has only one "unreactive" cysteine residue located at β112. The reaction of $HgCl_2$ with this group was insensitive to ligand (oxy, deoxy, CO) (Fig. 17). Activation energy for the reaction was roughly 8 Kcal/mole at 60 μM. Heme as computed from Fig. 16.

Rate II corresponding to the reaction of the first pair of the unreactive -SH groups of Hb A is apparently unaffected by the ligand state of the heme (Fig. 18). The activation energy for the reaction varies somewhat with hemoglobin concentration (Fig. 19); at 60 μM. heme it is 10.5 Kcal/mole. The effect of varying NaCl concentration (Fig. 20) is small, after corrections for actual $HgCl_2$ concentration.

The apparent congruence of ligand sensitivity and activation energy for the reaction of -SHβ112 of HbE and Rate II of Hb A would appear to justify the conclusion that Rate II of HbA in fact corresponds to the reaction of the β112 cysteine group. This

Fig. 16. Dependence of the apparent rate constant for Rate III on the NaCl concentration in 10mM Phosphate, pH 7.6 after correction for "real" $HgCl_2$ concentration.

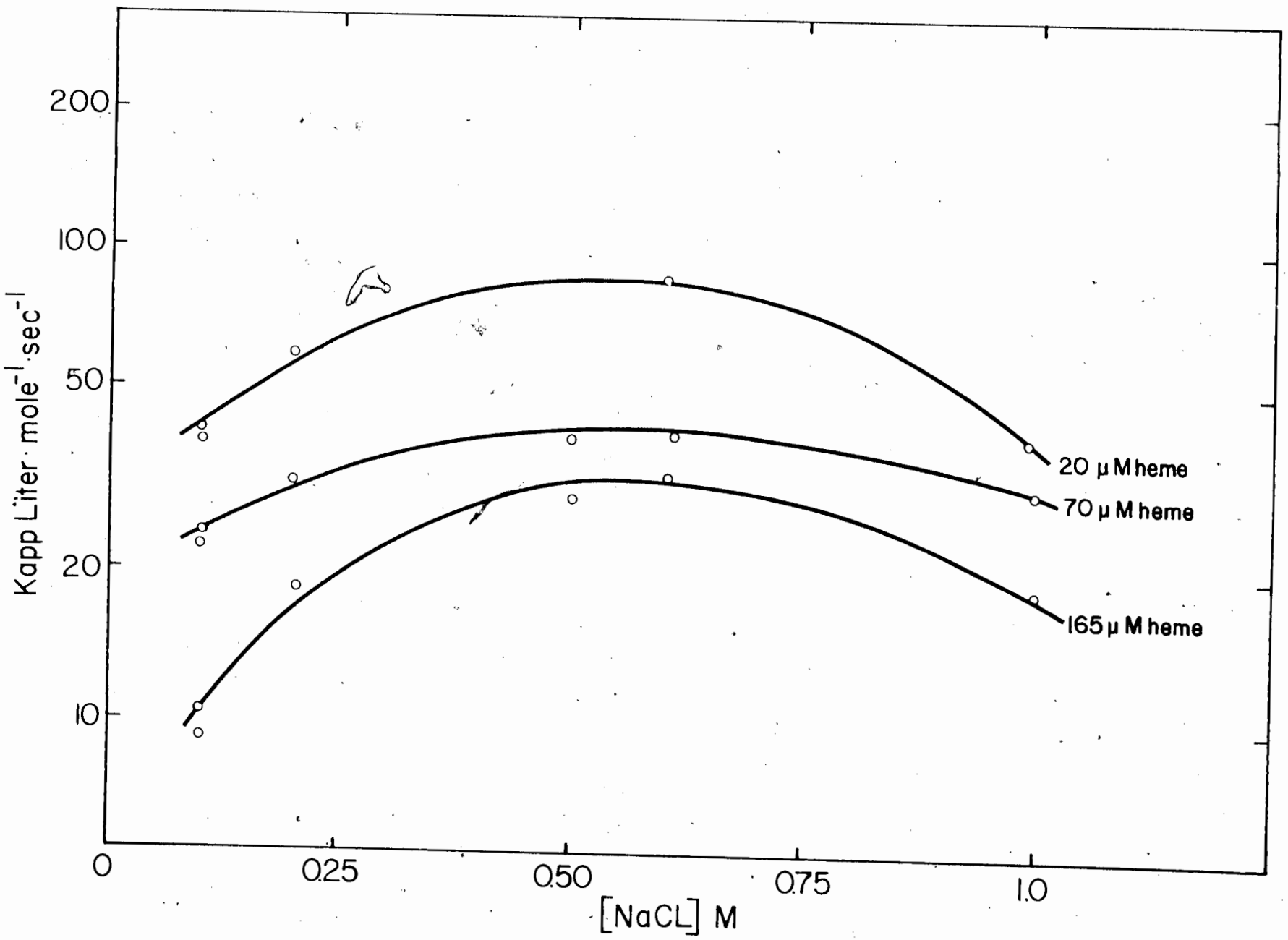

200
100
50
20
10
Kapp Liter · mole^{-1} · sec^{-1}
0
0.25
0.50
0.75
1.0
[NaCL] M
20 μ M heme
70 μ M heme
165 μ M heme

Fig. 17. The temperature dependence of the K_{app} for the reaction of -SHβ112 in the Hb E with $HgCl_2$. Hemoglobin concentration: 60 μM.heme in 100mM NaCl, 10mM Phosphate, pH 7.6. Data for oxy, deoxy and CO Hb is represented.

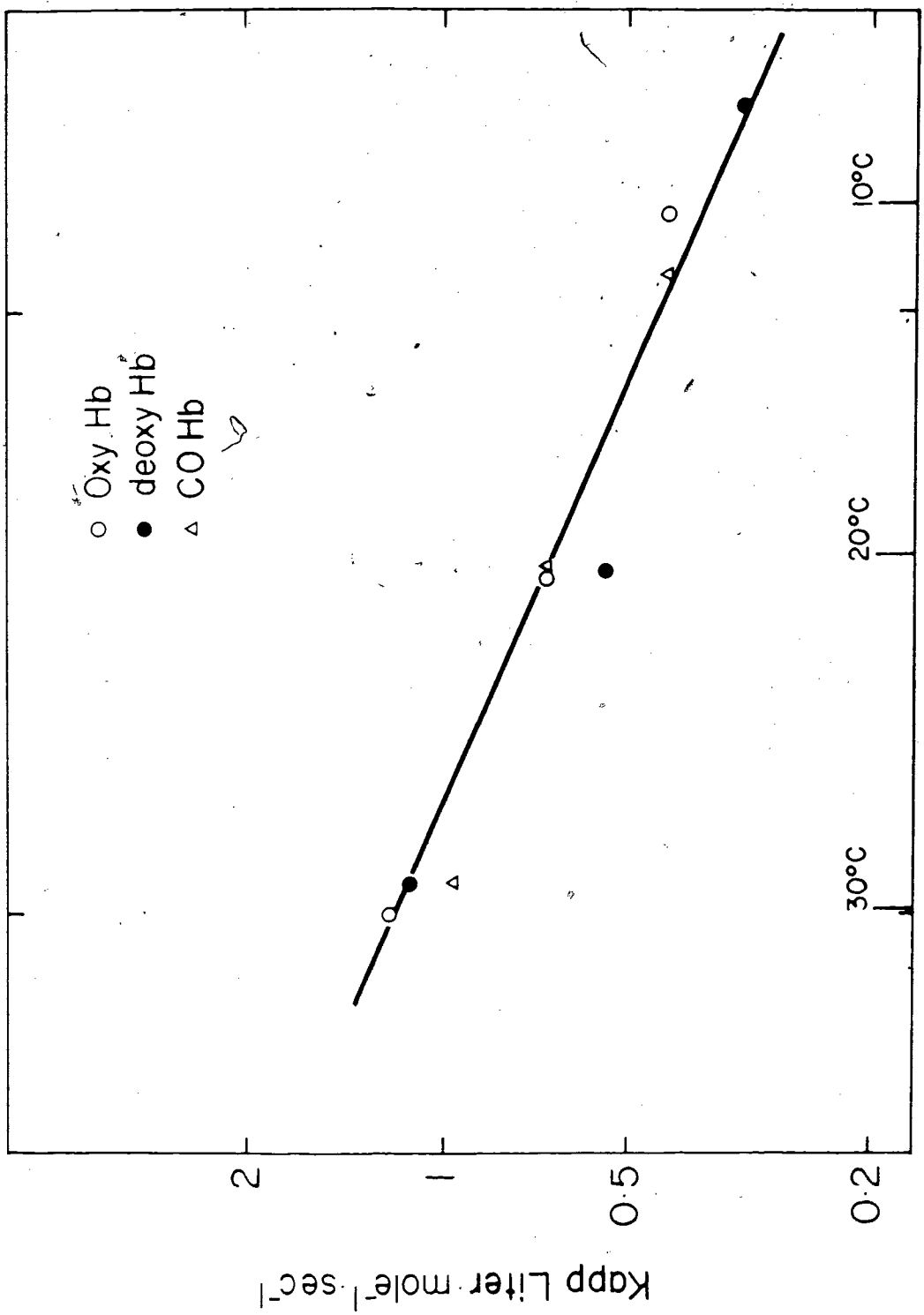

Kapp Liter mole⁻¹ sec⁻¹
2
1
0.5
0.2
○ Oxy Hb
● deoxy Hb
△ CO Hb
30°C
20°C
10°C
330
350
1/T x 10²

Fig. 18. The temperature dependence of K_{app} for Rate II (HbA). Hb concentration: 60 μM.heme in 100mM NaCl, 10mM phosphate, pH 7.6. Oxy Hb, deoxy Hb and CO Hb are represented.

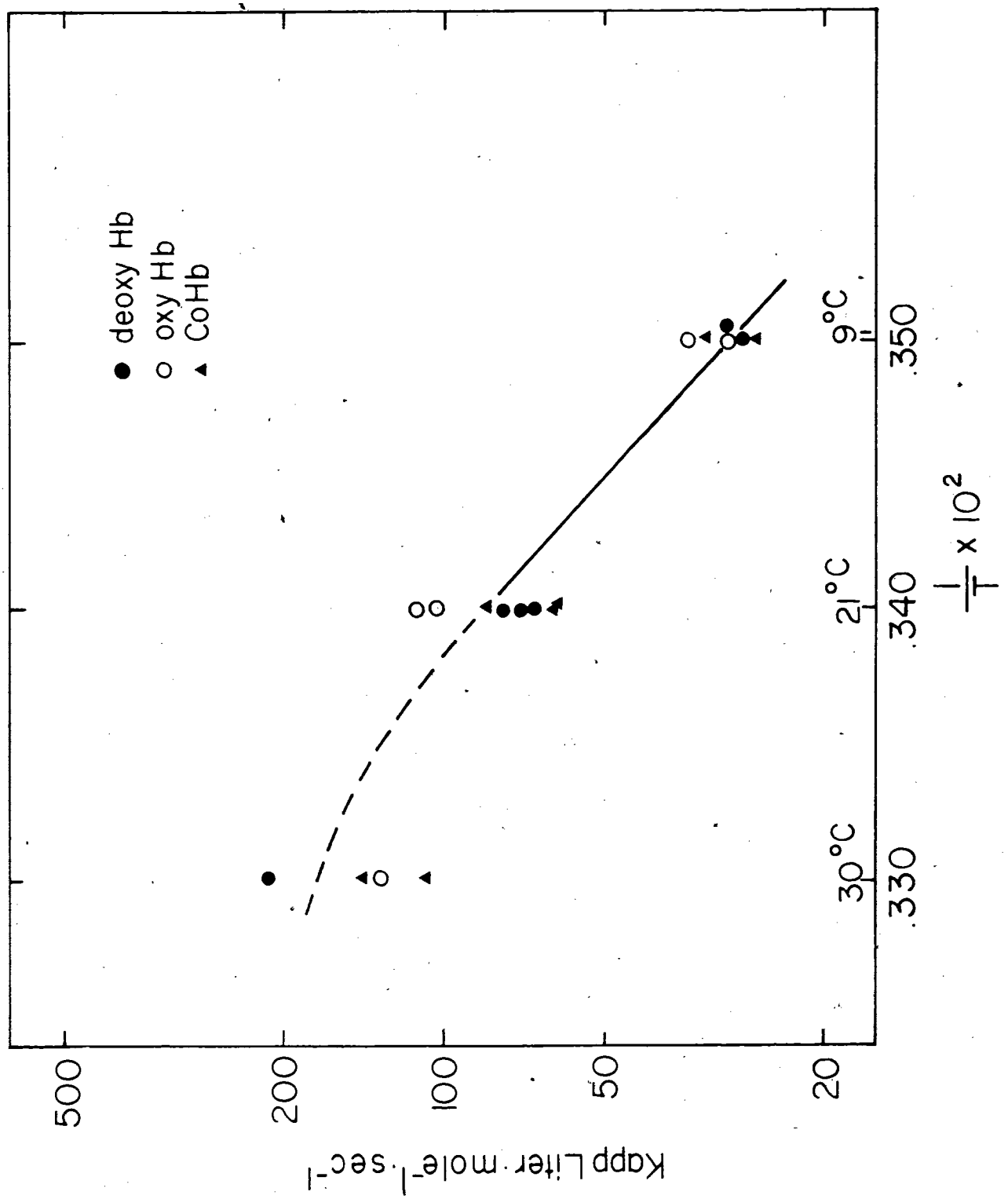

deoxy Hb
oxy Hb
CoHb
500
200
100
50
20
kapp Liter·mole⁻¹·sec⁻¹
30°C
21°C
9°C
.330
.340
.350
1/T x 10²

Fig. 19. The Hb concentration dependence of K_{app} for Rate II in 100mM NaCl, 10mM Phosphate, pH 7.6.

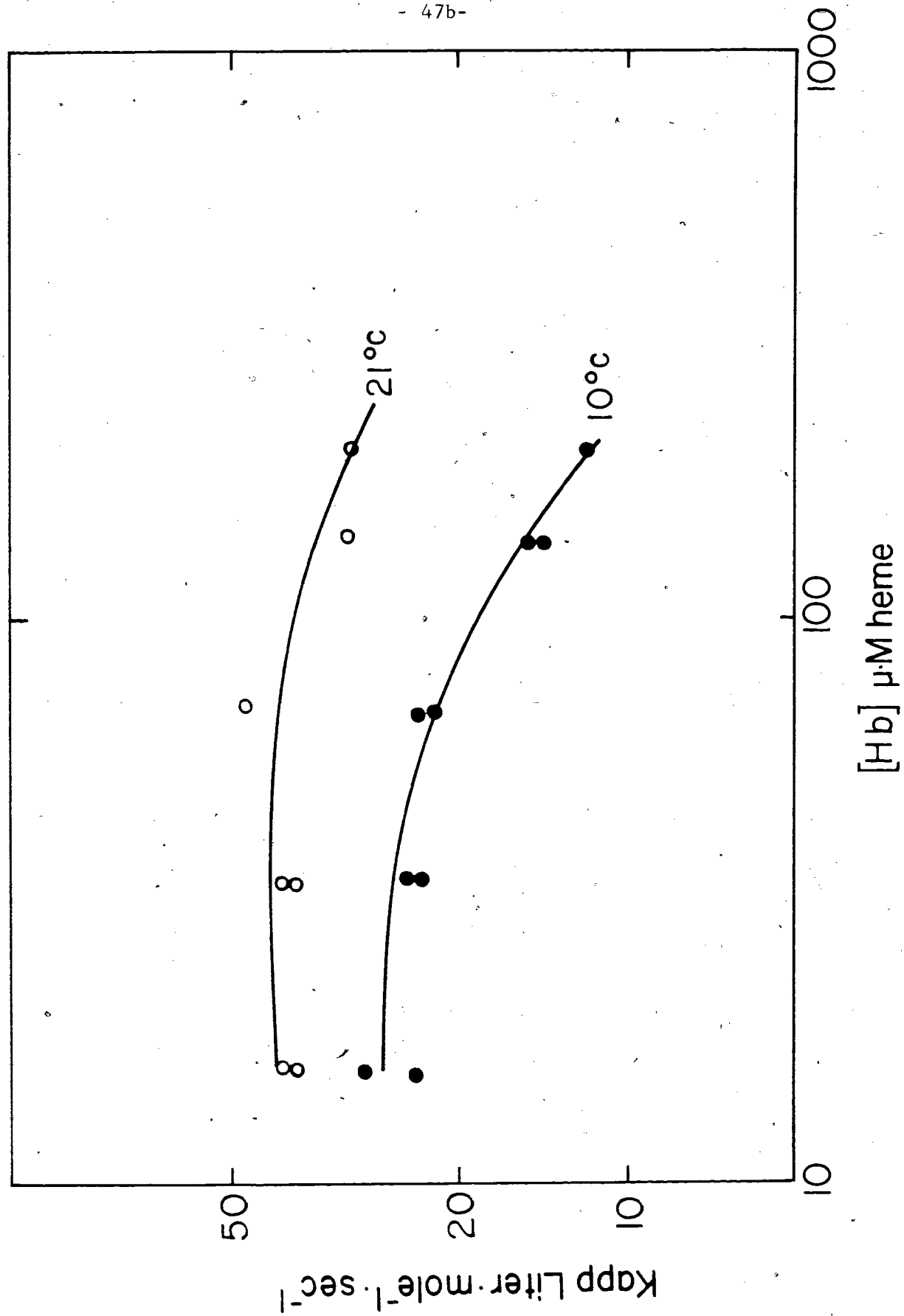

Kapp Liter·mole⁻¹·sec⁻¹
50
20
10
10
100
1000
[Hb] μM heme
21°c
10°c

Fig. 20. The NaCl concentration dependence of K_{app} for Rate II after correction for "real" $HgCl_2$ concentration. Hb concentration of 60 μM. heme in 10mM Phosphate, pH 7.6 at 21°C. Error bars represents the standard deviation on eight measurements.

Kapp Liter · mole^{-1} · sec^{-1}
500
200
100
0
0.25
0.50
0.75
[NaCL] M

conclusion is supported by the previously quoted results of Rosemeyer and Huehns (29) and others (36,37).

The α104 Cysteine Group

Preliminary results (Fig. 21) indicated that Rate III was insensitive to the ligand state of the heme. If these rate differences were, in fact, a reflection of allosteric interactions taking place between the α and β subunits, then the most useful information would be obtained from the apparent changes in the activation energy (E_a) of the reaction. The temperature dependence of K_{app} was determined for deoxy Hb and oxy Hb (Fig. 22) at an Hb concentration of 65 μM. heme.

The results were equivocal. The scatter in any given data set lead to an uncertainty in E_a in the order of $\pm$ 30%. A procedure was evolved in which Rate III was measured at two fixed temperatures over a wide range of Hb concentrations (Figs. 23, 24, 25). By measuring the distance between the two curves at a given concentration the E_a could be fixed within $\pm$ 5%.

Table I is a compilation of E_a's measured in this way.

Fig. 21. The ligand state dependence of Rate III. . This figure shows a lst order rate plot of reaction data for 50 μM. heme in 100mM NaCl, 10mM Phosphate, pH 7.6 at 20°C.

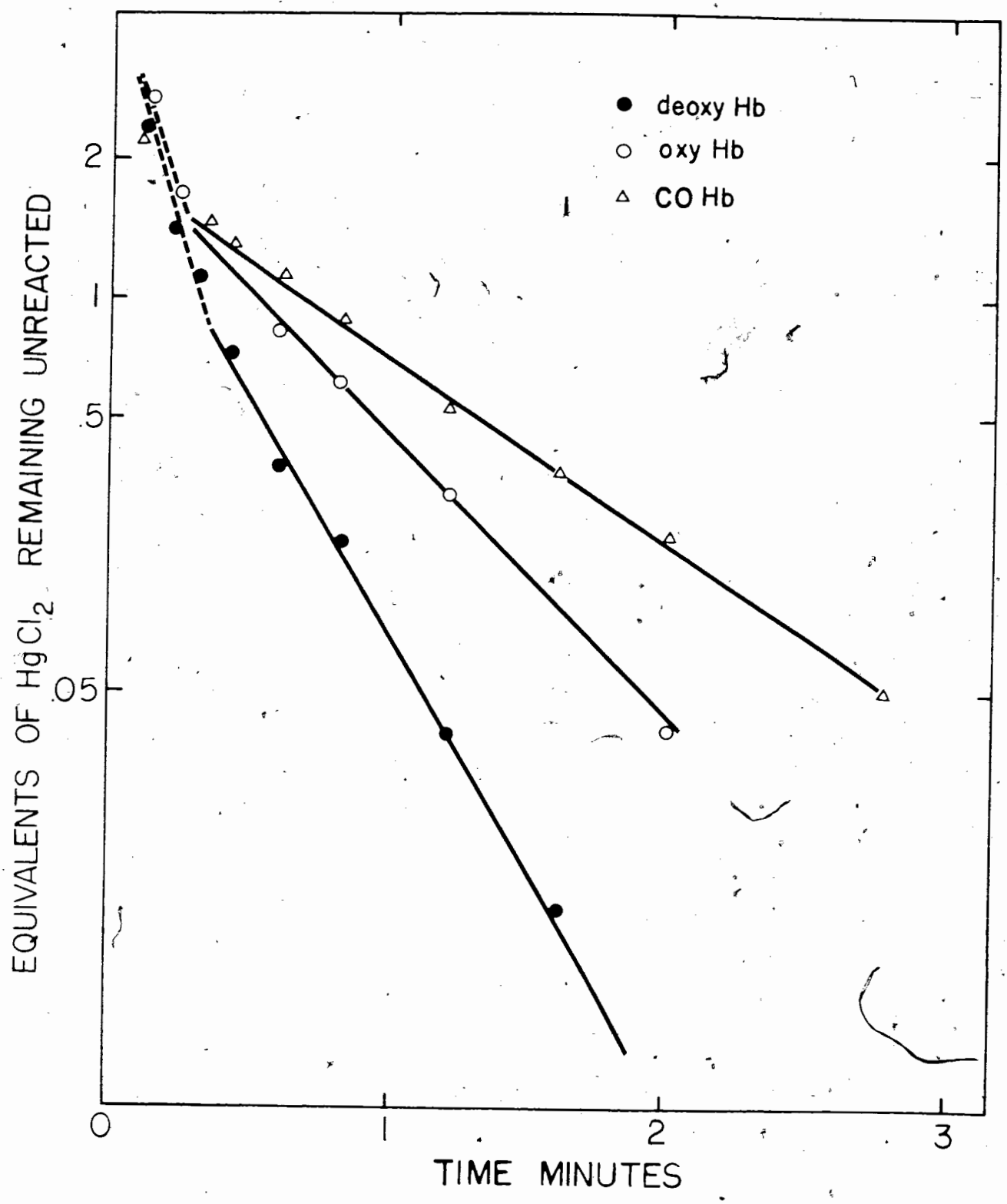

deoxy Hb
oxy Hb
CO Hb
EQUIVALENTS OF $HgCl_2$ REMAINING UNREACTED
2
1
.5
.05
0
1
2
3
TIME MINUTES

Fig. 22. Temperature dependence of K_{app} for Rate III; in 100mM NaCl, 10mM Phosphate, pH 7.6. The temperature dependence for K_{app} appears to be unaffected by the choice of reaction order. The two curves represent independent data sets, the lower curve being a first order analysis of Rate III in 55 μM.heme and the upper curve a second order analysis of Rate III in 65 μM.heme. Ligand effects are indistinguishable from the scatter.

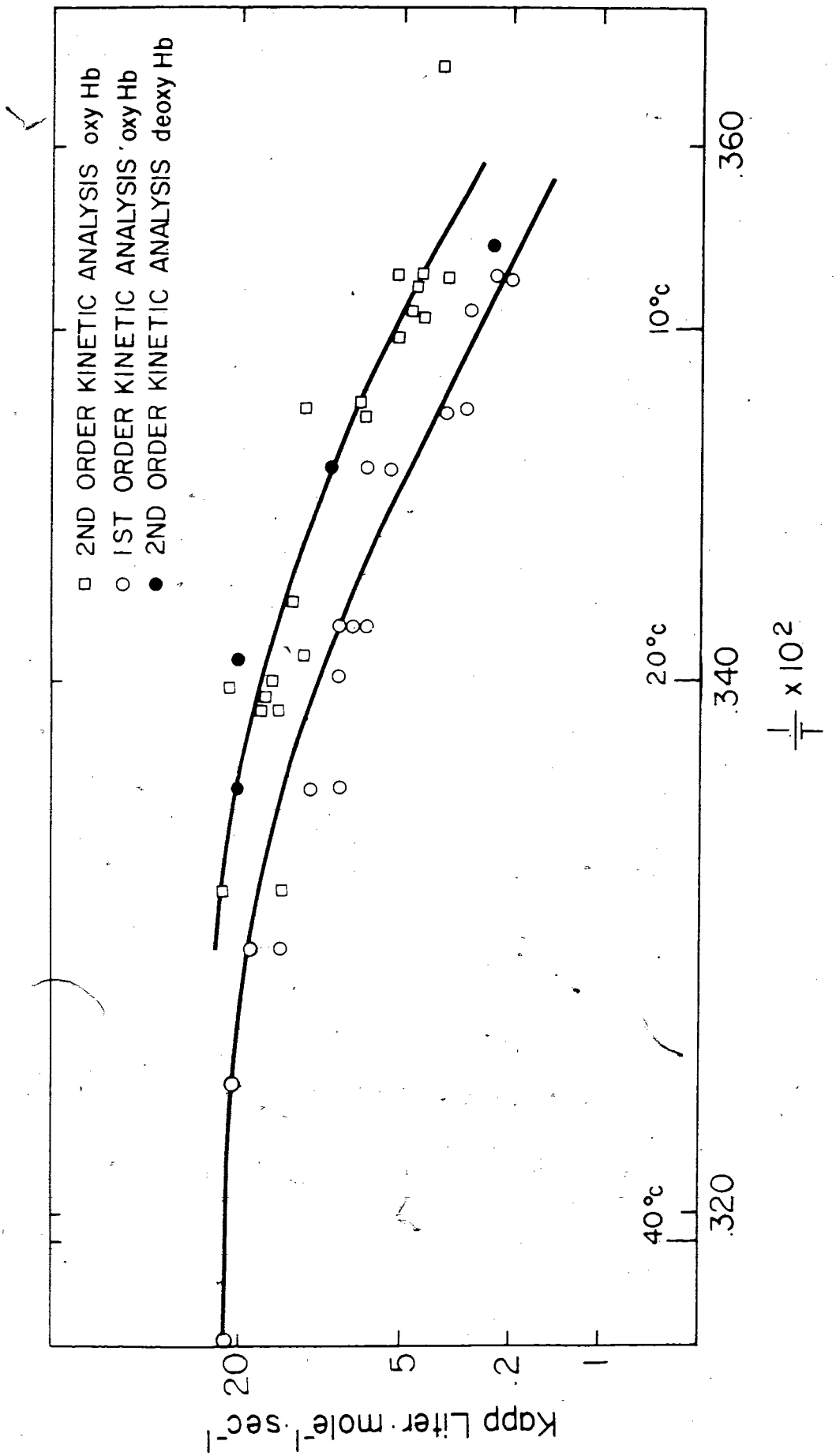

2ND ORDER KINETIC ANALYSIS oxy Hb
1ST ORDER KINETIC ANALYSIS oxy Hb
2ND ORDER KINETIC ANALYSIS deoxy Hb
Kapp Liter · mole⁻¹ · sec⁻¹
20
5
2
1
40°c
20°c
10°c
320
340
360
1/T x 10²

Fig. 23. The Hb concentration dependence of K_{app} for Rate III in 500mM NaCl, 10mM Phosphate, pH 7.6. Note that the E_a for the reaction which is proportional to the distance between the two curves, is concentration dependent.

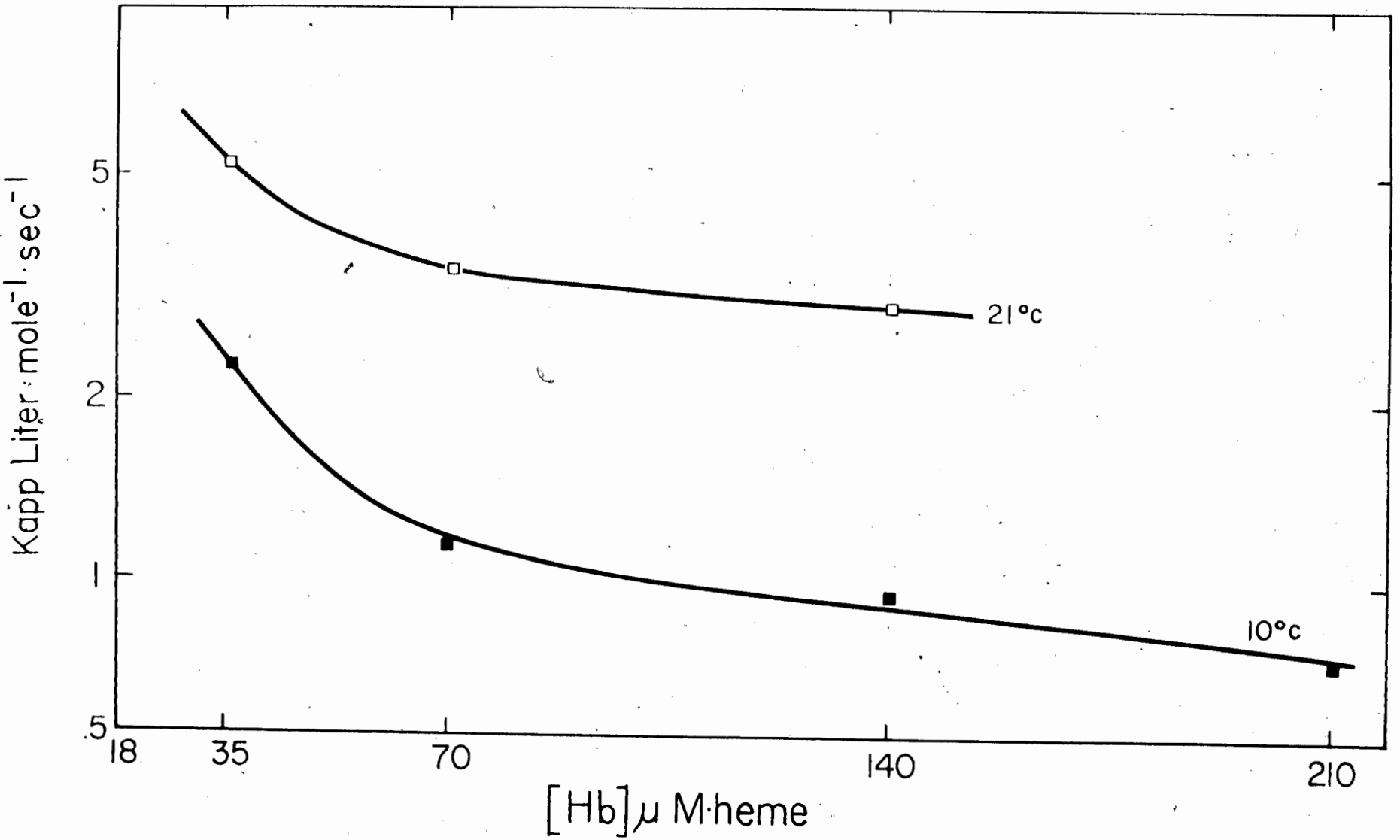

Kapp Liter·mole⁻¹·sec⁻¹
5
2
1
.5
18
35
70
140
210
[Hb] μ M·heme
21°c
10°c

Fig. 24. The Hb concentration dependence of K_{app} for Rate III in 100mM NaCl, 10mM Phosphate pH 7.6 2mM $CaCl_2$ plus 2mM $MgCl_2$. The slope of the curves is -1.

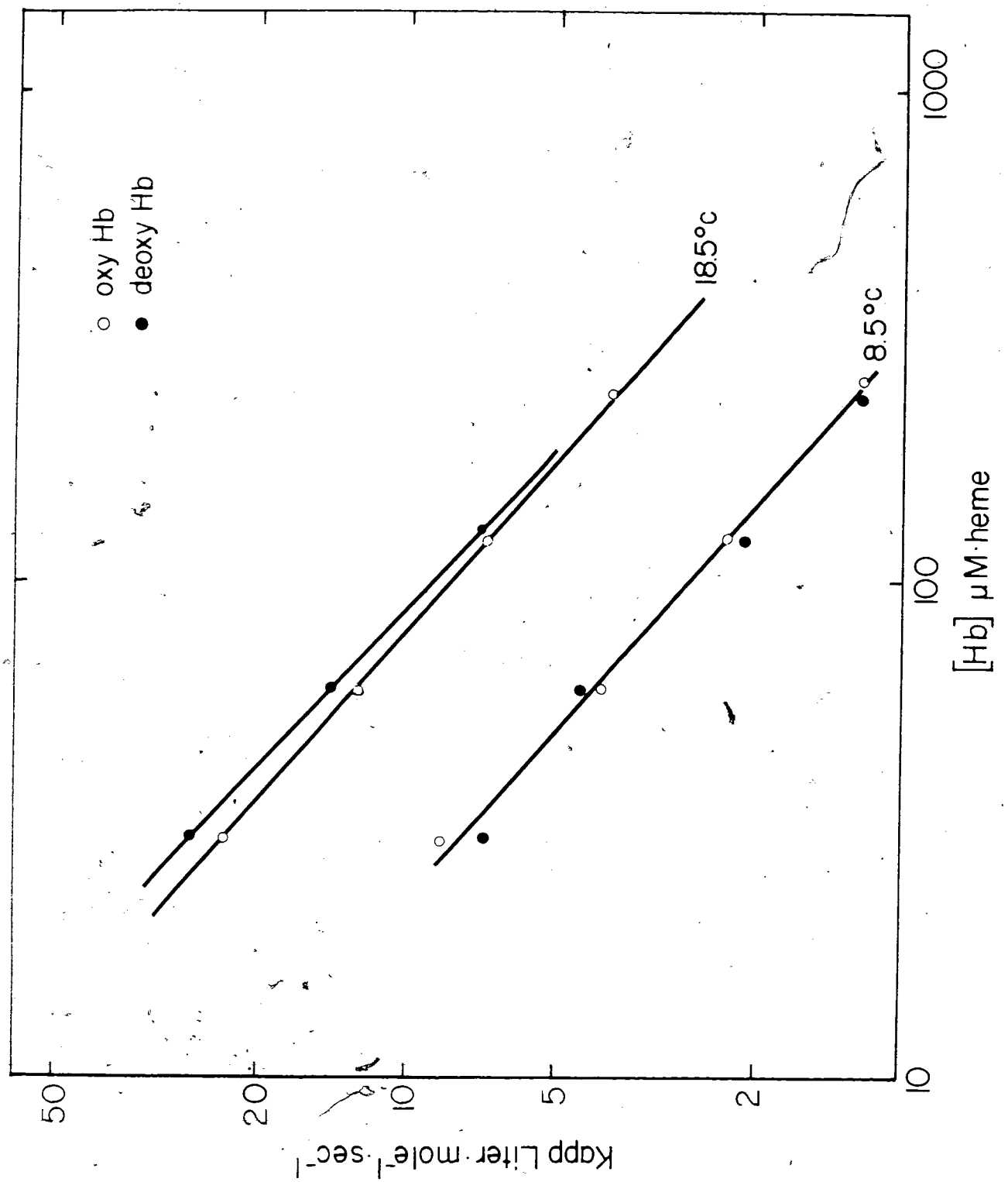

oxy Hb
deoxy Hb
18.5°c
8.5°c
kapp Liter·mole⁻¹·sec⁻¹
50
20
10
5
2
10
100
1000
[Hb] μM·heme

Fig. 25. The Hb concentration dependence of K_{app} for Rate III in 100mM NaCl, 10mM Phosphate, pH 7.6.

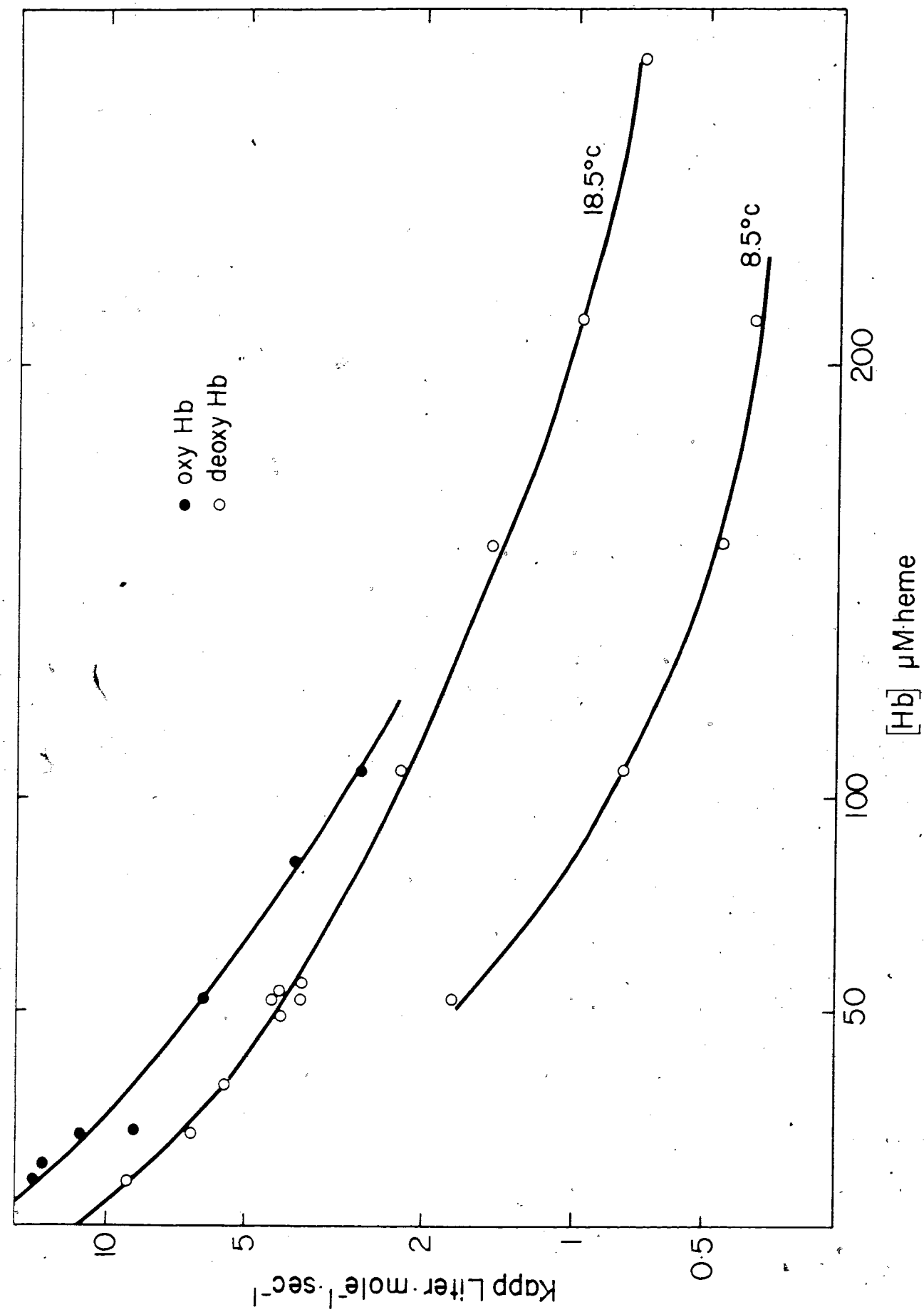

kapp Liter·mole⁻¹·sec⁻¹
10
5
2
1
0.5
50
100
200
[Hb] μM·heme
18.5°c
8.5°c
● oxy Hb
○ deoxy Hb

TABLE I

ACTIVATION ENERGIES FOR 65 μM.heme between 20°C and 10°C, Rate III.

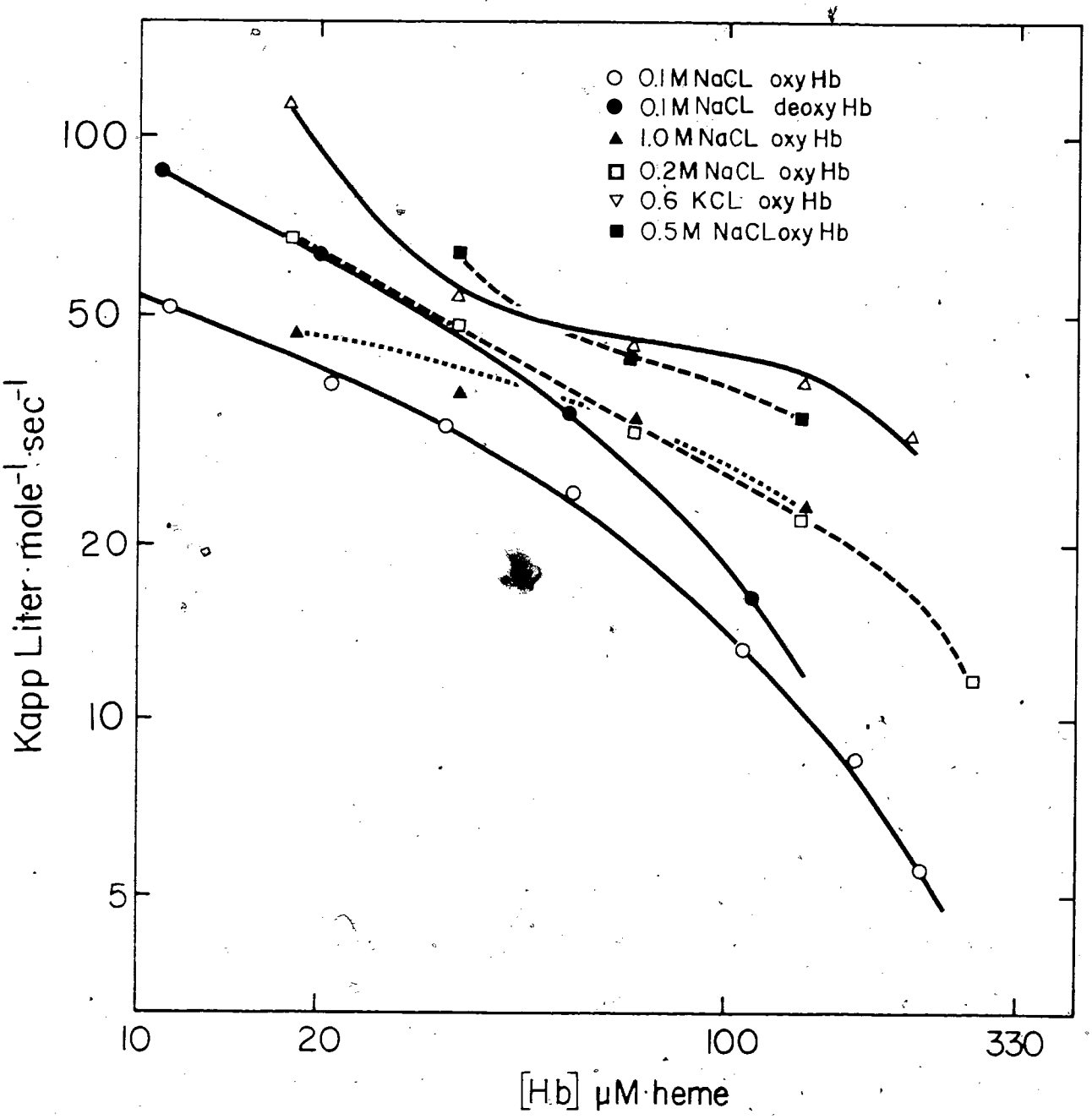
LIGAND	SALT	BUFFER SYSTEM	E_a
O_2	0.5M NaCl	10mM Phosphate pH 7.6	18.0 Kcal/mole
O_2	0.6M NaCl	" "	19.3
O_2	0.1M NaCl	" "	19.3
O_2	0.1M NaCl	" "	19.3
deoxy	2mM $CaCl_2$	" "	19.3
	2mM $MgCl_2$		

An important observation with respect to the E_a of this reaction was the fact that above 25 - 30°C the reaction has no apparent temperature coefficient. This can be seen in Fig. 22.

The dependence of the apparent rate constant on hemoglobin concentration is summarized in Fig. 26. In the region between 10 μM.heme and 100 μM.heme the slopes of most of the curves are close to -1/2. Beyond 100 μM.heme the slopes approach ~ -1 i.e. $K_{app} \propto \frac{1}{Hb}$. Apparent rates for deoxy Hb only diverge from oxy Hb at relatively low Hb concentrations, < 50 μM.heme (Figs. 24, 25).

The addition of divalent cations to the titration medium had a rather striking effect on the relationship between K_{app} and Hb concentration (Fig. 28). As well as altering the concentration dependence, the presence of Ca^{+2}, Mg^{+2} in millimolar amounts tended

Fig. 26. The Hb concentration dependence of K_{app} for Rate III in 10mM Phosphate, pH 7.6 at various salt concentrations. All data corrected to 20°C, corrected for "real" $HgCl_2$ concentrations.


○ 0.1 M NaCL oxy Hb
● 0.1 M NaCL deoxy Hb
▲ 1.0 M NaCL oxy Hb
□ 0.2M NaCL oxy Hb
▽ 0.6 KCL oxy Hb
■ 0.5 M NaCL oxy Hb
100
50
20
10
5
Kapp Liter·mole⁻¹·sec⁻¹
10
20
100
330
[Hb] μM·heme

to reduce any rate differences between oxy Hb and deoxy Hb (Fig. 24).

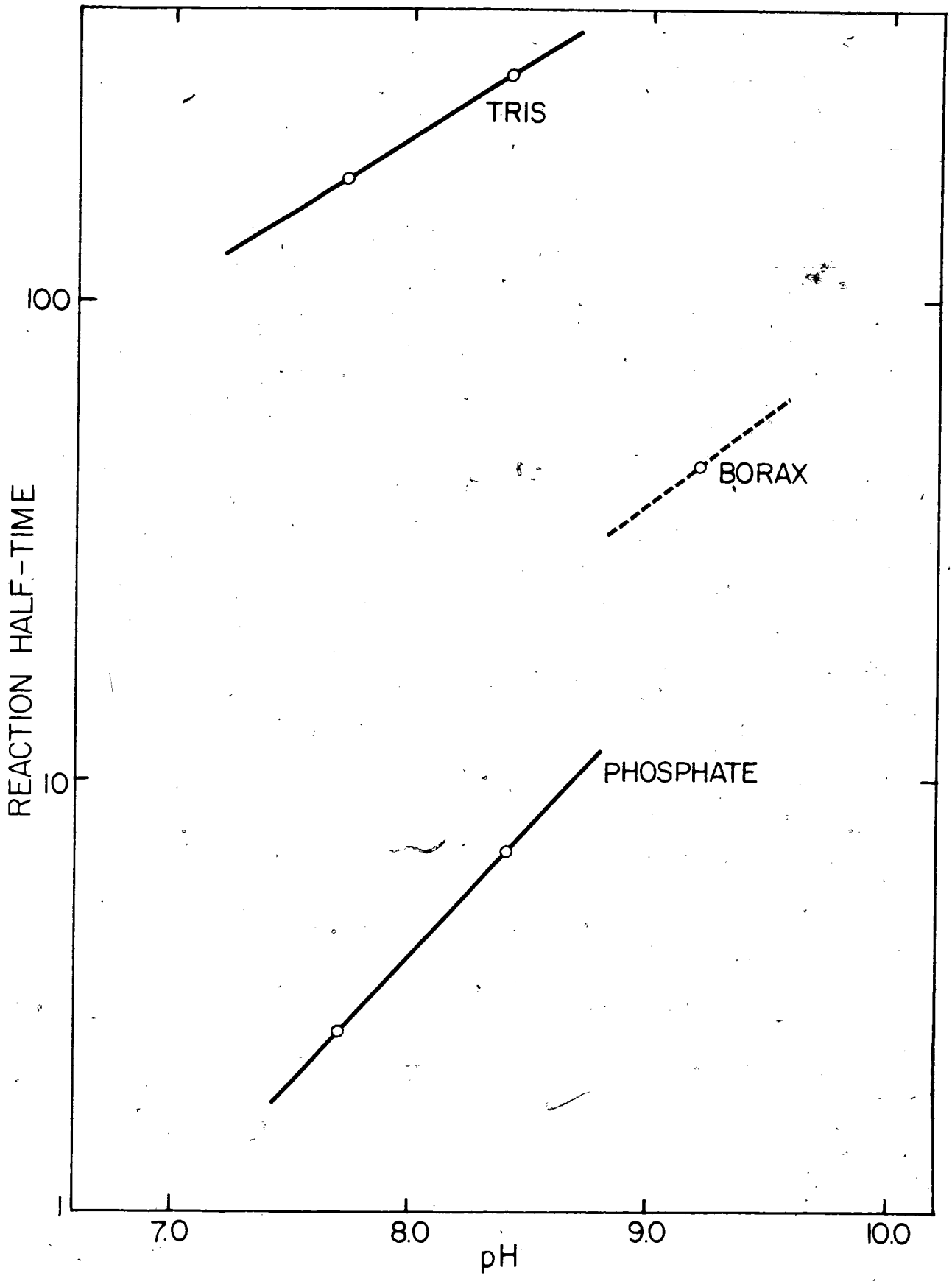
The effects of 2,3-DPG on Rate III were explored. No differences were seen between Hb stripped of 2,3-DPG, unstripped Hb and stripped Hb with a 2.5 molar equivalents of 2,3-DPG added (both oxy Hb and deoxy Hb reacted in Buffer (P)). The reaction rate is strongly dependent on pH and on the nature of the buffer system. The observations are summarized in Fig. 27.

The failure to find any appreciable reflection of ligand effects in the E_a of the reaction indicated that a probe of allosteric subunit interactions based on E_a differences would not be feasible unless there were to be a tenfold increase in experimental resolution. This did not appear to be possible with the apparatus being used. The fact remained, however, that there was a definite difference in the reaction rates of oxy Hb and deoxy Hb (Fig. 25). In the absence of detectable E_a differences, these rate differences could not be reflecting subunit interactions, but they may have resulted from changes in the solution attributable indirectly to the absence or presence of a ligand on the Hb molecule. There will be discussion of this point in the next section.

One possibility which immediately suggested itself was that only the monomeric or dimeric form of Hb was reactive with $HgCl_2$. In such a case the hemoglobin concentration dependence for the apparent rate constant ought to reflect the ratio of dimer or monomer to total Hb, ϕ , as discussed in the Theoretical section.

Although there is ample literature on the effects of pH,

Fig. 27. The effects of pH and of the nature of the buffer system on Rate III reaction half time. Hb concentration 60 μM.heme in 100mM NaCl, plus 100mM Buffer.


TRIS
BORAX
PHOSPHATE
100
10
1
REACTION HALF-TIME
7.0
8.0
9.0
10.0
pH

salts, and ligand on the association constants for Hb (48,59-66), there is no information pertinent to the situation in which the -SHβ93 of Hb A has been reacted with $HgCl_2$. It is possible to fit some of the data in Fig. 25 to a dimer - tetramer equilibrium scheme and to assign an equilibrium constant in retrospect; however, there did not appear to be any subunit equilibrium scheme which would fit more than a segment of any one of the concentration dependence curves. To enumerate observations in support of a "dimer" type hypothesis:

1) Apparent reaction rates decreased with increasing Hb concentration. This would correspond to the decreasing value of $\frac{D}{HBT}$ as Hb increased.

2) The effect of increasing salt concentrations was to increase the apparent reaction rate constant which would correspond to increasing dimerization of Hb (63).

There are several observations which could not be reconciled with any reaction scheme which invoked differential reaction rates for different orders of subunit association.

1) Deoxyhemoglobin shows a higher apparent rate constant than does oxy Hb. Unmodified deoxy Hb is known to be primarily tetrametric (60). If the tetramer of Hb were more reactive, then the apparent rate constant should also increase with increasing Hb concentration, which it does not do.

2) The Hb concentration dependence of the apparent rate constant in Buffer Ⓒ is proportional to $[Hb]^{-1}$ (Fig. 24).

This relationship would not fit <u>any</u> subunit equilibrium situation.

III. THE DIFFUSION COEFFICIENT FOR MERCURIC CHLORIDE IN HEMOGLOBIN SOLUTIONS

Introduction

Since Rate III appeared to have no substantial temperature coefficient above 25°C (Fig. 22), it was proposed that the reaction of $HgCl_2$ with the unreactive -SH groups might be diffusion limited.* Since the diffusion rates of $HgCl_2$ might have a direct bearing on the observed concentration, ligand etc., effects, the relative values for D_{Hg} were measured in Hb solutions.

From Stock (19) the amperometric limiting current can be expressed as:

$$i_{lim} = K^1 nCD_o^z$$

The instantaneous sensitivity of the amperometric apparatus S, will be equivalent to $\frac{di_{lim}}{dC}$. If the increment in C is small then D will be independent of C and

$$S = \frac{di_{lim}}{dC} = K^1 nD_o^z \qquad (17)$$

* In retrospect, it would appear that the facts mitigate against such a conclusion. The rate constant for a diffusion controlled reaction should be in the order of 10^9 $M^{-1}.sec^{-1}$ whereas the rate constants for the reaction in question are in the order of 10^1 $M^{-1}.sec^{-1}$ (71). Nonetheless the experimental data collected in pursuit of this dubious hypothesis, shows a high degree of correlation between D_{Hg} and K_{app}. Evidently there is a strong relationship between the two which bears further investigation. As the nature of this relationship is for the moment unresolved, the historical context for the experimental information appears to be the best vehicle for its presentation.

D_o^z can then be determined from measurements of S. The actual value of the exponent is not well known. Nightengale (67) determined $z = \frac{2}{3}$ for a rotating coaxial Pt electrode but this relationship only held for certain ions. Levich (68) determined theoretically that the value for a rotating disc electrode, with laminar flow as in the co-axial electrode, should be $z = \frac{2}{3}$. For a dropping electrode he determined $z = \frac{1}{2}$ as previously determined by Ilkovic (69). In the case of the D.M.E. the reduced value of z resulted from convection at the surface of a tangentially expanding mercury droplet. For a radial rotating Pt electrode there has been no theoretical treatment because of the difficulty in dealing with turbulence. Ferrett and Phillips (20) experimentally approximated the value of z at $\frac{1}{3}$ for a rotating radial electrode with the trailing edge insulated.

At best it can be concluded that for the experiments under discussion performed with a bare, radial rotating Pt electrode the value of z should fall somewhere in the range between .5 and .3.

RESULTS AND DISCUSSION

Sensitivity was determined as a function of Hb concentration in titration media Buffer (P) and Buffer (C) . Mercuric chloride concentration was 4 molar equivalents in excess of the six -SH groups per Hb_4 (i.e., 10 equivalents of $HgCl_2$ were added to each hemoglobin solution). It can be seen from Fig. 9 that this is a sufficient excess to avoid any non-linearity resulting from the

weak binding of Hg^{+2}.

The results were significant. The congruency between the S vs Hb curves in Fig. 29 and the K_{app} vs [Hb] in Fig. 28 is not likely to be coincidental.

The values of $D_{Hg}{}^{z}$ have been determined. The value of z supposedly lies between 0.5 and 0.3. Taking an intermediate value for the exponent z as z = 0.4 and dividing apparent reaction rates by the computed value of D_{Hg}; the net effect is that K_{corr} becomes more or less independent of Hb concentration as shown in Fig. 30. Though this manipulation would appear to involve a judicious manipulation of "Finagle's Constant" (in the form of the exponent z), the limits on the values which z can assume are well defined. Even if either of the two extreme values are used, K_{corr} remains almost independent of Hb concentration.

Further, the relationships of D^{z} and K_{app} to Hb are idiosyncratic but identical. This in itself is a strong argument that the apparent rate constant and diffusion coefficient are directly related.

Apparent rate differences between oxy Hb and deoxy Hb entirely disappear when the diffusion coefficient is taken into account. Sensitivities measured in deoxy Hb solutions diverge from sensitivities in oxy Hb at lower concentrations by an amount which exactly eliminates any ligand induced changes in the apparent rate constant (after correction for D_{Hg} using $z = 0.4$). The divergence is smaller in Buffer Ⓒ and at high Hb concentrations. On the other hand

Fig. 28. The effect of divalent cations on the Hb concentration dependence of K_{app} for Rate III. (P) is the reaction in 100mM NaCl, 10mM Phosphate, pH 7.6, (C) is the same solution plus 2mM $CaCl_2$, 2mM $MgCl_2$. Temp. = 20°C.

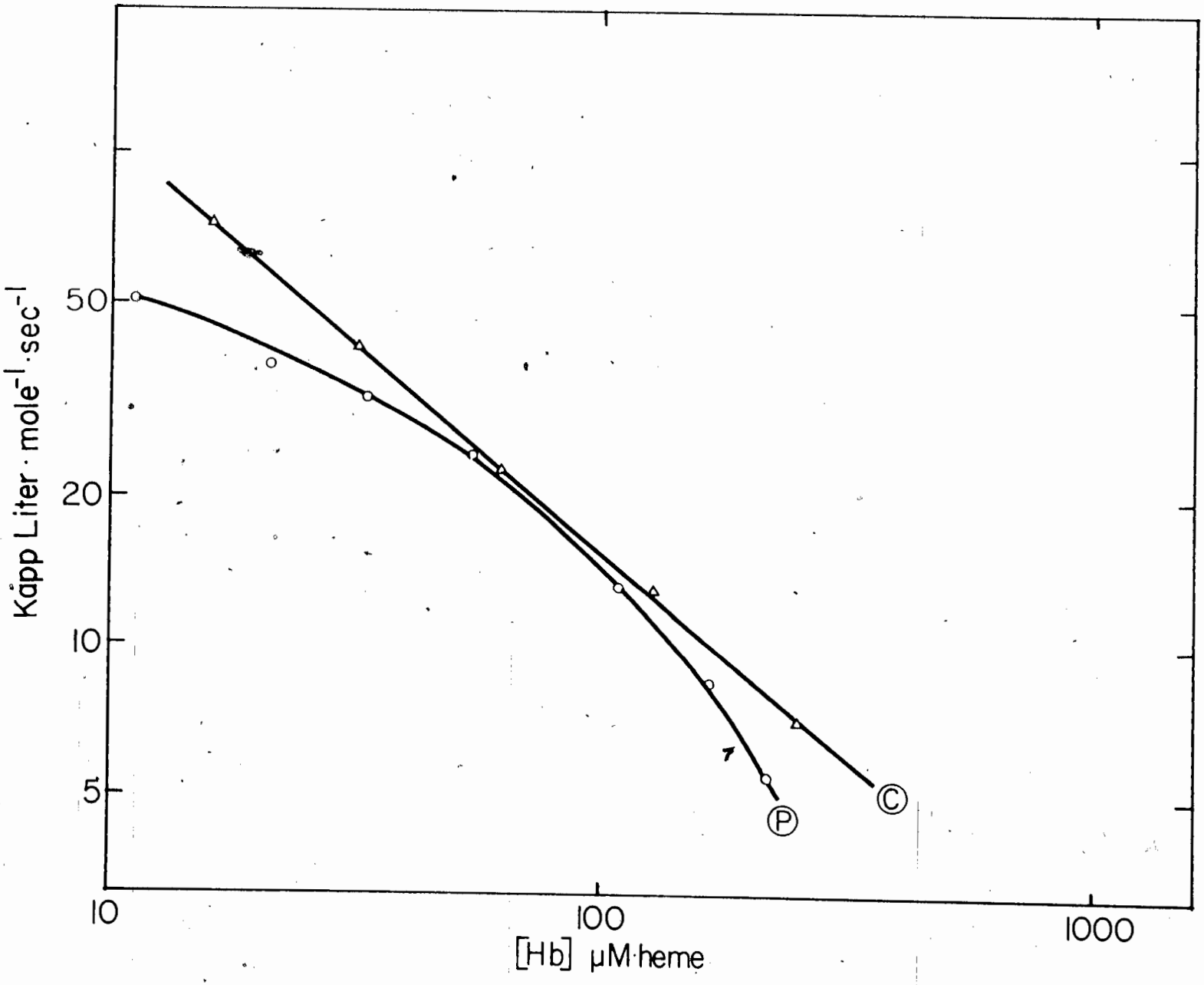

Kapp Liter · mole⁻¹ · sec⁻¹
50
20
10
5
10
100
1000
[Hb] μM heme
Ⓟ
Ⓒ

Fig. 29. Sensitivity of the mercurimetric apparatus as a function of Hb concentration at 20°C. Notations (P) and (C) have the same meaning as in Fig. 27.

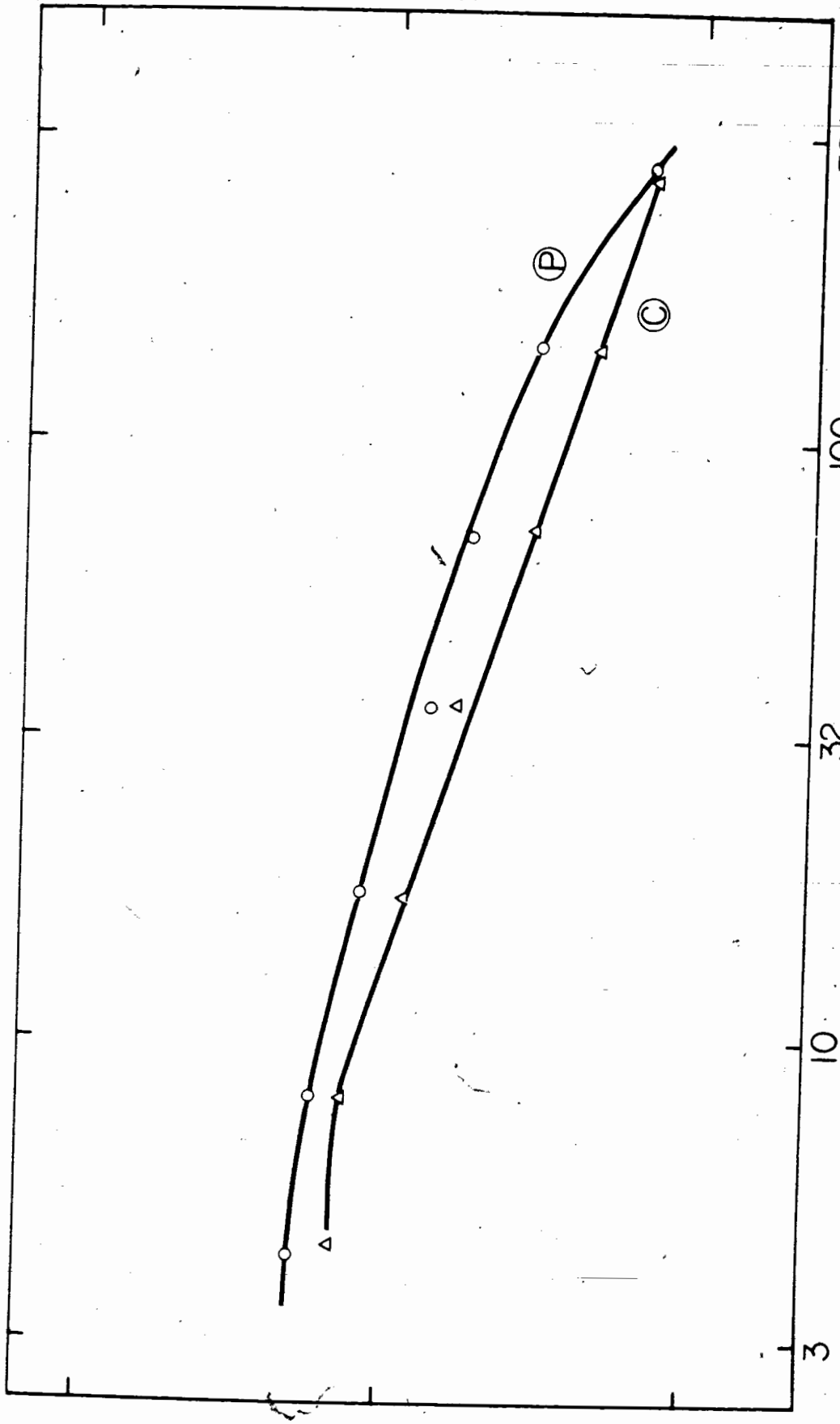
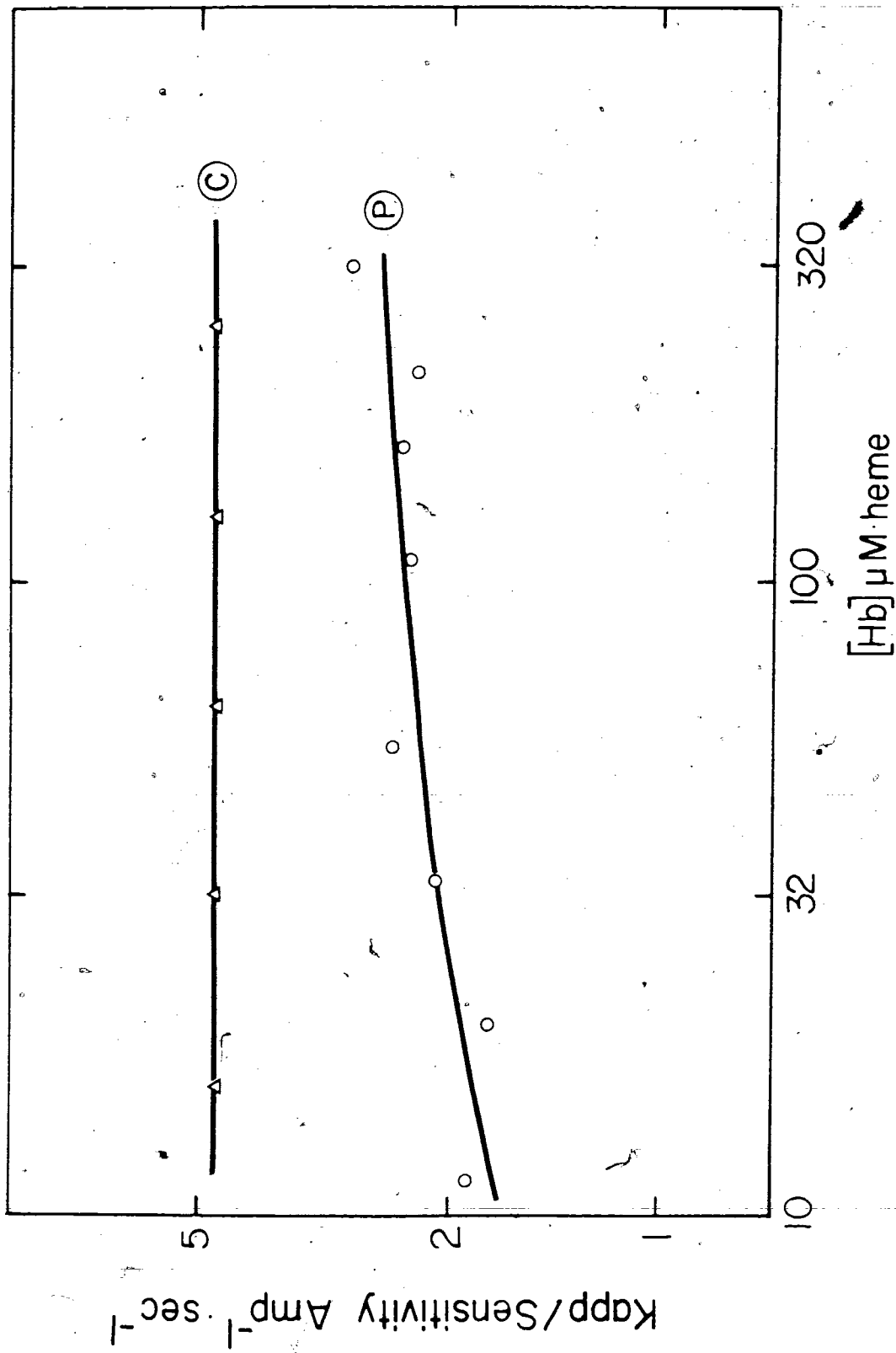

Sensitivity Amp·liter·mole⁻¹
3
10
32
100
320
[Hb] µM heme
Ⓟ
Ⓒ

Fig. 30. The Hb concentration dependence of K_{app} for Rate III after correction for D_{Hg}. The reaction conditions and notations are identical to those in Fig. 27.


Kapp/Sensitivity Amp^{-1} sec^{-1}
5
2
1
10
32
100
320
[Hb] μM heme
C
P

the sensitivity does not have a large enough temperature coefficient to eliminate the apparent activation energies. S changes by ~20% over the range from 21°C to 8°C. This would make the listed E_a values high by ~30%. However the compensation has not been made since this alteration could possibly result from temperature dependent electrode characteristics rather than changes in D_{Hg}.

The effect of NaCl concentration on S has not been thoroughly investigated but from available data the reduction in S in going from 0.2M NaCl to 1.0M NaCl amounts to ~20%. The relationship in Fig. 16 therefore is probably still valid.

Although the correction of the apparent rate constants for D_{Hg} would explain the observations of Part II one is left with the equally puzzling question of the causes for the apparent changes in D_{Hg} with ligand, and Hb concentration.

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