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Hemoglobin from the Common Murre Uria aalge shows two components; a major component (Hb A) comprising about 80% of the total hemoglobin, and a minor component comprising about 20%. Stripped Hb A shows a high oxygen affinity which is greatly reduced by the presence of IHP. The presence of ATP also reduces the affinity of Hb A, but it's effect differs in several other parameters. Stripped Hb B shows a moderate oxygen affinity but is essentially unaffected by the presence of IHP. NaCl increases the affinity in both cases. The Bohr effects and the cooperativities of the two components are compared under various conditions. The function of a multiple hemoglobin system in diving birds such as the murre is discussed in this thesis.

THE STRUCTURE AND FUNCTION OF HEMOGLOBIN FROM THE COMMON MURRE *URIA AALGE*

by
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A THESIS

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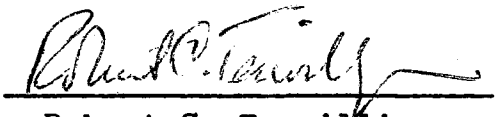
APPROVED: 

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TABLE OF CONTENTS

TABLES AND FIGURES

Tables

Figures

INTRODUCTION

Avian hemoglobins have not been studied to the degree that one might expect considering the widespread popularity of this group of animals. Although many comparative studies have been carried out, most detailed studies of avian hemoglobin structure and function have been done on domestic species. There are several characteristics of the Common Murre Uria aalge which make its hemoglobin a desirable subject for study. 1. The murre is a wild bird eliminating the possibility of artificially inbred characteristics. The domestication of animals could allow the propagation of characteristics which would prove disadvantageous in the wild. 2. The murre is a proficient diver among birds, spending up to 3 minutes underwater. 3. The murre is an Alcidae, a family of birds not usually available for this type of study.

The murres spend nearly their entire lives at sea, coming to land only briefly to mate and rear their young (Tuck, 1961). About 20 days after hatching the young murres leave the land and follow their parents out to sea. Adult murres weight between 800 and 1200 grams (Johnson, 1944) and consume between 24 and 27% of their body weight per day (Sanford and Harris, 1967). The

murre is a wing propelled diving bird which captures its food underwater. The diet consists mainly of free swimming fish and cephalopods, indicating that the murres actively pursue their prey at mid water levels (Pearson, 1968; Varoujean, pers. comm.). There is evidence that they may dive as deep as 120 ft. and remain submerged for up to three minutes (Varoujean, pers. comm.). When compared to the marine mammals the diving ability of the murre seems rather modest. However, among the birds the murre is a quite proficient diver, second perhaps only to the penguins. During feeding, murres have been observed to dive repeatedly for extended periods of time with relatively brief recovery periods between dives. The hemoglobin, therefore, may assist the respiratory needs of the animal both during a dive, and during the recovery period between dives.

The diving ability of an animal is dependent on its ability to withstand hypoxia. Lenfant et al. (1970) listed three factors which may contribute to the degree of hypoxic endurance in diving animals. These factors are 1. metabolic adjustments, 2. functional properties of the blood and oxygen storage factors, and 3. oxygen conservation mechanisms. Diving mammals usually have blood with the following properties: high buffering capacity, high hematocrit, and a hemoglobin with a high

affinity for oxygen and a low Bohr effect (Lenfant, 1969; Wells and Brennan, 1979). The Adélie penguin shows functional characteristics similar to those of diving mammals (Lenfant et al., 1969). However, other diving birds such as the murre must meet the requirements of flight as well as diving. Thus murre whole blood has a high hematocrit and high buffering capacity, as needed for diving, but a large Bohr effect and relatively low affinity, as required for flight (Lenfant et al., 1969). In mammals, functional adaptations of the hemoglobin have been shown to make only a limited contribution to the animal's ability to withstand hypoxia (Lenfant et al., 1970; Simon et al., 1974). The role of hemoglobins in avian diving is unknown.

The structure of the hemoglobin of a number of avian species has been investigated. Avian adult hemoglobin has been shown to have one to three components. Usually there are two components (Saha and Ghosh, 1965), one major component comprising about 75% of the hemoglobin and one minor component comprising about 25% (Vandecasserie et al., 1971). Moss and Hamilton (1974) demonstrated that in the chicken these components have different α chains whereas the β chains are very similar in structure.

Recently there has been much interest in the ability

of certain organic phosphates to modify the oxygen equilibrium properties of hemoglobin. Human erythrocytes contain 2,3 diphosphoglycerate (2,3 DPG) and adenosine triphosphate (ATP) (Benesh and Benesh, 1967; Chanatin and Curnish, 1967). These compounds were shown to reduce the oxygen affinity of hemoglobin considerably. Rapoport and Guest (1941) determined that the main organic phosphate in avian erythrocytes is inositol hexaphosphate (IHP). Later Steward and Tate (1969) showed that this organic cofactor was in fact myoinositol pentaphosphate (IP_5). Vandecasserie et al., (1976) demonstrated that the effect of IHP on hemoglobin oxygenation is only slightly greater than that of IP_5. Thus IHP is still used in many studies since it is more readily available. Mission and Freeman (1972) reported that in early chicken embryos ATP was the major organic phosphate present with IHP levels increasing in the last few days before hatching. In the Peking duck embryo, 2,3 DPG is present as well as ATP (Borgese and Lampert, 1975). The embryonic presence of 2,3 DPG in chickens was confirmed by Isaacks and Harkness (1975). Shortly after hatching 2,3 DPG is no longer detectable (Bartlett and Borgese, 1976). Ochiai et al. (1972) demonstrated that IHP decreased the oxygen affinity of hemoglobin up to a 1-1 Hb-IHP ratio, while ATP gradually decreases the affinity well beyond

the 1-1 Hb-ATP ratio. Tyuma et al. (1971) suggested that the mechanism of action by which IHP lowers oxygen affinity in human hemoglobin differs from the mechanism for ATP and 2,3 DPG.

The functional aspects of the major and minor components of chicken hemoglobin were studied by Huisman et al. (1964) and Huisman and Schillhorn van Veen (1964). The minor component showed a higher affinity than the major component in phosphate buffer. Vandecasserie (1971) also showed a higher oxygen affinity for the minor component in chickens. In the turkey, pheasant, and duck the stripped minor component has a lower affinity than the stripped major component. The major component, however, is much more sensitive to the effects of IHP in reducing its oxygen affinity so that with IHP present the major component has a lower affinity than the minor component (Vandecasserie et al., 1973). The physiological significance of these observations are not known.

In the present study the structure and function of murre hemoglobin was investigated. Structural experiments were designed to determine the number of hemoglobin components present in the murre red blood cell, and to elucidate any subunit differences between those components. Purified murre hemoglobin components were investigated by oxygen binding experiments to determine any functional

differences. The effects of allosteric modifiers on the oxygen binding properties of the major and minor components of murre hemoglobin were also investigated. This data may help determine the purpose of multiple hemoglobins in avian erythrocytes. The effects of the two major organic phosphates, IHP and ATP, on the major component of murre hemoglobin were compared.

MATERIALS AND METHODS

Samples of hemoglobin from the adult Common Murre Uria aalge were collected at sea from birds being killed for other purposes. The birds were killed by shotgun and were retrieved as quickly as possible. On deck the body cavity was opened and blood collected from the hepatic artery, the ventricle, or the jugular vein. Collection was accomplished by puncture of the heart or veins with an 18 guage needle and 10cc syringe containing a few grains of heparin. Samples were then placed in vials, sealed and kept on ice until return to shore.

The red blood cells were then centrifuged for five minutes at 1,100 g in a Sorvall RC2-B automatic refrigerated centrifuge and the clear supernatant discarded. The resulting pellet of red blood cells was resuspended twice in 0.5 M NaCl and centrifuged as a washing procedure. After the second wash the centrifuge tube containing the pellet was sealed with parafilm and frozen for storage.

The samples were removed from the freezer and thawed in cold 0.05 M Bis-Tris HCl buffer with 0.1 M NaCl titrated to pH 7.0. The pellet was gently agitated with a stainless steel spatula. Upon thawing the cells lysed

and the solution became a dark red. The sample was then centrifuged in the refrigerated centrifuge for 10 minutes at 12,100 g. The supernatant was saved as a hemoglobin solution and the pellet re-extracted with fresh buffer as above. The two resulting supernatant solutions were combined for use.

A column of Sephadex G-25 measuring 20 cm high and 2.5 cm in diameter was equilibrated with 0.05 M Bis-Tris HCl buffer with 0.1 M NaCl titrated to pH 7.0. The hemoglobin sample was then passed through this column to strip it of salts, phosphates, and any other impurities (Berman et al., 1971). Hemoglobin treated in this manner will be designated stripped hemoglobin in the following text.

Isoelectric focusing (IEF) was performed on a sample of murre blood to determine if multiple hemoglobins were present. Carbon monoxide was bubbled through the stripped murre Hb to produce COHb which was used for IEF. A 7.5% acrylamide solution was prepared with a 1-1 ratio of ampholine 3.5-10 and ampholine 5-8 (LKB products) as the gel buffer. The IEF was run on a Bio-Rad model 300 A gel electrophoresis cell. The lower buffer was 0.01 M phosphoric acid, and the upper buffer was 0.02 N NaOH. The focusing was run at 150 V maximum and was water cooled at 14° C. Since COHb is red, no stain was

necessary to see the bands. The gels were carefully removed from the tubes and scanned with a Zeiss PMQ-II spectrophotometer at 540 nm. The distance that the bands had migrated was measured. The gels were then cut into 5 mm lengths and each segment placed in a small sample (2 ml) of distilled water overnight. The pH of each sample was then measured to determine the isoelectric point of the hemoglobin fractions.

A column of DEAE cellulose (Sigma Chemical Co.) was prepared by washing DEAE cellulose first with 0.5 M HCl and then with 0.5 M NaOH. It was then rinsed with distilled water and equilibrated with 0.01 M Tris HCl buffer pH 8.0. The column was poured to about 7 cm height by 2 cm diameter and flushed with several volumes of buffer. A sample of stripped murre Hb was dialyzed exhaustively against pH 8.0 Tris HCl buffer and added to the top of the column. When all freely moving sample had passed off of the column, the sample which had bound to the top of the column was released by adding a gradient of 0 to 0.1 M NaCl to the column buffer. Fractions were collected with a Gilson fraction collector. Fraction volumes were measured and absorbances measured with a Zeiss PMQ-II spectrophotometer. The percent of each component could then be estimated from the volume and relative concentrations. Separation of the fractions on a

DEAE ion exchange column was used as a preparatory technique for experiments involving the separated fractions.

The subunit structure of the hemoglobin fractions was investigated using 6.25 M urea gel electrophoresis. Fractions were first dialyzed versus 0.1 M ammonium bicarbonate and then dehemed in a separatory funnel with acid methyl ethyl ketone (Teale, 1959). The aqueous portion containing the globin was then dialyzed against large volumes of distilled water, and the sample lyophilyzed. The sample was then incubated for 12 hours at room temperature in 10 M urea which had been previously deionized with Amberlite Mb 1, and 1% 2-mercaptoethanol before electrophoresis. Electrophoresis was carried out in a 5% gel which was 6.25 M in urea (Panyim, 1969). Lower and upper buffers were 5% glacial acetic acid. Gels were pre-electrophoresed at 2 ma per tube for 3½ hours. The electrophoresis was run at 2 ma per tube for 3 hours. The gels were stained with comassie blue stain and then destained with 10% glacial acetic acid.

A sample of stripped murre Hb was passed through a calibrated Sephadex G-100 column to determine its apparent molecular weight. The column buffer was 0.05 M Tris HCl with 0.01 M NaCl and 0.01 M $MgCl_2$. The column had been calibrated with bovine serum albumin (M_r = 68,000), ∝ chymotrypsinogen A (M_r = 25,000), and sperm whale

myoglobin (M_r = 17,800). Samples were collected in a Gilson fraction collector and the elution volume measured.

Hemoglobin which was used in oxygen binding experiments was first stripped on Sephadex G-25 and then separated on DEAE as above. The resulting samples were then concentrated by vacuum dialysis until the solution was a very deep red. They were then dialyzed against four changes of 250 ml each 0.01 M Tris HCl pH 8.0. This insured that the concentration of salts in the sample did not increase along with the hemoglobin concentration. To prepare a sample for oxygen binding under specific conditions, a buffer prepared of 0.01 M Tris HCl with the appropriate salts, was titrated to the desired pH. One or two drops of the concentrated Hb solution was added to a 5 ml sample of the buffer solution. This hemoglobin solution was then readjusted to the proper pH using small amounts of buffer at a higher or lower pH as needed.

The oxygen affinity of the major component (Hb A) was measured to determine the effect of the following: different pHs, inositol hexaphosphate (IHP), NaCl, both IHP and NaCl together, different temperatures, and different buffer concentrations. For equilibrium studies involving IHP or ATP, the concentration of these organic phosphates was 0.001 M; when NaCl was used the

concentration was 0.1 M. All experiments involving potential allosteric modifiers except those for temperature and buffer concentration were carried out over a pH range of 7.0 to 8.0 to determine the Bohr effect for hemoglobin with each modifier. The temperature effect was studied at pH 7.4 on stripped Hb A in 0.01 M Tris HCl buffer. The temperature range was 10° C to 30° C. The effect of buffer concentration was studied at pH 7.4 and 20° C.

Samples of the minor component (Hb B) were prepared as with Hb A except for a dialysis with 2 changes of 500 ml 0.01 M Tris HCl before concentrating by vacuum dialysis. This dialysis was intended to remove the 0.1 M NaCl which was necessary to elute Hb B off of the DEAE ion exchange column. Oxygen equilibrium curves for the minor component were determined in order to assess the effects of pH, IHP, and NaCl. The pH range and the concentration of the modifiers were the same as with Hb A.

Oxygen equilibrium curves were determined by the method of Benesh et al., (1965) with a Ziess PMQ-II spectrophotometer equipped with a constant temperature cell holder. Absorbances were measured at 540, 555, and 575 nm.

RESULTS

Isoelectric focusing of hemoglobin from the adult Common Murre shows two peaks (Fig. 1). The large peak represents the major component of murre hemoglobin (Hb A) and travels the least distance. The smaller peak represents the minor component (Hb B) and migrates farther during the electrophoresis. The isoelectric point determined by the average of three runs is 8.2 for the major component and 7.95 for the minor component. A control of human hemoglobin shows a large band at pH 7.3 and a small band at pH 7.7, which is in general agreement with values listed elsewhere (Drysdale et al., 1970; Antonini and Brunori, 1971).

The elution pattern of murre Hb on DEAE is shown in Fig. 2. Testing the separated fractions Hb A and Hb B by isoelectric focusing gives a single peak for Hb A, and a single peak for Hb B. A 1-1 mixture of the two components gives two peaks of equal size. This indicates that the fractions are in fact different and that DEAE is a suitable separation technique.

The volume and absorbancies of the fractions from the DEAE column (Fig. 2) show that Hb A makes up about 80% of the total hemoglobin and Hb B makes up about 20%.

Elution of adult murre hemoglobin on a G-100 gives an apparent molecular weight of 53,000. This value approximates the molecular weight of other vertebrate tetrameric hemoglobins.

The results of urea gel electrophoresis are shown in Fig. 3. Hb A separates into two approximately equal bands which electrophorese fairly close together. Hb B, however, presented some unexpected problems in that it would not dissolve well in 10 M urea with 2-mercaptoethanol. Bands were seen only when large aliquots of the incubation mixture were electrophoresed. As a result the bands were diffuse and thus inconclusive. However, there appear to be two equal bands from Hb B. The combination of Hb A and Hb B produced three bands.

In dilute buffer stripped Hb A shows a very high oxygen affinity above pH 7.8 and below pH 7.2, with a slightly lower oxygen affinity (P_{50} = 2 mmHg) between these two points (Fig. 4). The lowest affinity occurs about pH 7.4. The stripped pigment shows no Bohr effect between pH 7.2 and 7.6. There seems to be a slight Bohr effect between pH 7.6 and 8.0 and a slight reverse Bohr effect between pH 6.9 and 7.2. The Hill coefficient (h) varies between 2.0 and 3.0 (Fig. 5). The highest cooperativity occurs between pH 7.3 and 7.6. The physiological pH of murre blood is 7.4 (Lenfant et al, 1969).

The h at pH 7.4 shall be used to compare cooperativity of Hb solutions. The h at 7.4 for stripped Hb A is 2.9 (Table 1).

In the presence of 1 mM NaCl the oxygen affinity of Hb A is slightly higher than that of stripped Hb A (Fig. 6). However, a pH dependency is now evident with a ϕ value of -0.40 (Table 1). The degree of cooperativity for Hb A with NaCl does not seem to be affected by pH between pH 7.2 and 7.8 (Fig. 7). The presence of NaCl does increase the cooperativity of the pigment above pH 7.8 and below pH 7.2. The h at 7.4 does not differ greatly from the pigment without the NaCl (Table 1).

The addition of 1mM inositol hexaphosphate (IHP) to the binding buffer results in a considerable decrease in the oxygen affinity of Hb A (Fig. 8). At pH 7.4 the P_{50} of Hb A with IHP is increased by a factor of 10 above stripped Hb A. The Bohr effect is relatively weak (ϕ = -0.20). Though this Bohr effect is greater than that of the unmodified pigment, it is less than the value for Hb A with NaCl. The Hill coefficients for Hb A are generally lowered by the presence of IHP. The h at pH 7.4 is decreased to 1.75. Fig. 9 shows the h values for Hb A with IHP decreasing with decreasing pH. The slope of the h vs pH graph is shown in Table 1, and can be used to compare the sensitivity of the heme-heme

interactions to pH.

Since IHP and NaCl each have some effect on the oxygen binding properties of the major component of murre hemoglobin, these two modifiers were combined to see what the total effect would be (Fig. 10). Throughout the range of pHs examined, the combination of IHP and NaCl produces a P_{50} lower than that of IHP alone (Fig. 11). The presence of NaCl does increase the Bohr effect over that of IHP alone (Table 1). The h values with IHP and NaCl present are slightly lower than those for IHP alone (Fig. 12), and more sensitive to pH (slope h vs pH = 0.87).

The effects of 1 mM adenosine-5'-triphosphate on the major component of murre hemoglobin is shown in Fig. 13. At this concentration ATP is similar to IHP in that it increases the P_{50} of Hb A considerably. Hb A with ATP, however, shows a high Bohr effect (ϕ = -0.60). Fig. 14 shows Hb A with ATP having a fairly high cooperativity, with h at pH 7.4 being 2.7. The slope of h vs pH for Hb A with ATP is 0.63 (Table 1).

When ATP and NaCl are both present in the binding buffer (Fig. 15) the affinity of Hb A is higher than with just ATP present. Hb A with ATP and NaCl has a P_{50} at pH 7.4 of 13.8 mmHg, and a Bohr effect factor of -0.46. The Hill coefficient of Hb A with ATP and NaCl remains

moderately high (Fig. 16). The h and pH 7.4 is 2.6. Hb A with ATP and NaCl shows a moderate sensitivity of heme-heme interaction to pH, with a slope h vs pH of 0.45.

The stripped minor component of murre hemoglobin (Hb B) shows a much lower affinity for oxygen than stripped Hb A (Fig. 17). The P_{50} at pH 7.4 of stripped Hb B is 17.4 mmHg. The pigment has only a slight Bohr effect as shown by the ø value of -0.27 (Table 1). Fig. 18 shows stripped Hb B having low cooperativity which is only slightly sensitive to pH.

When 0.1 M NaCl is added to stripped Hb B the affinity is generally increased and the Bohr effect is more pronounced (Fig. 19). The heme-heme interaction is reduced but the sensitivity of the cooperativity to pH is increased (Fig. 20).

In the presence of 1 mM IHP, stripped Hb B shows no sensitivity to pH (Fig. 21). The P_{50} remains relatively high with a value at pH 7.4 of 15.1 mmHg. The heme-heme interaction is also fairly low and insensitive to pH (Fig. 22).

The oxygen affinity of stripped murre Hb A increases with an increase in the concentration of the Tris HCl buffer at pH 7.4 as shown in Fig. 23. The cooperativity of the subunits also increases with increasing buffer concentration (Fig. 23).

Murre Hb A shows an increase in affinity with a decrease in temperature (Fig. 24). The Hill coefficient shows no change with temperature (Fig. 24).

DISCUSSION

The structural data for murre hemoglobin shows no unusual variations from other avian hemoglobins. Both isoelectric focusing and DEAE ion exchange showed a major component comprising about 80% of the hemoglobin and a minor component comprising about 20%. This is consistant with the general pattern found in avian hemoglobins. The major component (Hb A) and the minor component (Hb B) appear to have one subunit in common and one subunit that differs. This agrees with the subunit structure reported by Moss and Hamilton (1974). However, the results presented here are inconclusive and warrant further investigation. It is of interest that Hb B did not dissolve well in 10 M urea with 2-mercaptoethanol. Future studies on the structure of the minor component of murre hemoglobin may elucidate a case for this phenomenon.

The high affinity of stripped murre Hb A in dilute buffer is not without precedent in other birds. The stripped major component of chicken Hb has an oxygen affinity very similar to that of murre Hb A (Vandecasserie et al., 1971; Ochiai et al., 1972). The slight reverse Bohr effect below pH 7.3 is probably a result of the low

ionic strength of the buffer. The addition of NaCl decreases the affinity of stripped Hb A below pH 7.3, but does not have a similar effect on the slight normal Bohr effect of stripped Hb A above pH 7.6. This indicates that the increased affinity of stripped Hb A at low and high pH are probably distinct effects with different causes. Stripped Hb A also shows a reduced h value below pH 7.3, an effect which disappears with the addition of NaCl. No such reduction in cooperativity of stripped Hb A is seen above pH 7.6.

The minor component of chicken hemoglobin has a higher affinity for oxygen than the major component (Vandecasserie et al., 1971). This relationship is reversed in the turkey, pheasant and duck (Vandecasserie et al., 1973). Murre Hb is similar to the latter condition in that stripped Hb B has a much lower affinity than stripped Hb A (Fig. 25). Hb B also differs from Hb A in showing a lower cooperativity.

IHP acts as a potent modifier when added to stripped Hb A (Fig. 11). The P_{50} at pH 7.4 is increased by an order of magnitude over stripped Hb A without IHP. However, Hb B is relatively insensitive to the presence of IHP showing very little change in affinity (Fig. 26). The presence of IHP does allow a slight Bohr effect in Hb A, but with Hb B and IHP there is no Bohr effect.

Kilmartin, (1973) demonstrated a decrease in the cooperativity of human Hb A with the addition of IHP. Similarly IHP causes a reduction in the heme-heme interaction of murre Hb A (Table 1). However, the already low cooperativity of murre Hb B is not reduced further by the addition of IHP. The cooperativity of Hb A with IHP present shows a fairly strong sensitivity to pH while the cooperativity of Hb B is insensitive to pH.

The addition of 1 mM ATP to murre Hb A causes a decrease in affinity of the same general order of magnitude as 1 mM IHP. However, the effect of ATP on Hb A differs from that of IHP in several other characteristics. Hb A with 1 mM ATP produces a stronger Bohr effect than Hb A with 1 mM IHP. Also 1 mM ATP does not lower the cooperativity as does 1 mM IHP. The sensitivity of the cooperativity to pH is high in the presence of either organic phosphate. Apparently the mechanism by which IHP affects Hb A differs from that of ATP. This is in agreement with the findings of Tyuma et al., (1971) for human Hb A.

NaCl has the general effect of increasing the affinity of murre hemoglobins. Over most of the pH range the P_{50} of stripped Hb A is reduced slightly by the addition of NaCl, with the exception of the low pHs mentioned earlier. When NaCl is added to murre Hb B the

affinity increase is more distinct. NaCl also increases the affinity of Hb A in the presence of organic phosphates. The change in P_{50} when NaCl is added to Hb A with IHP is approximately equal to the change when NaCl is added to Hb A with ATP (Table 1). The bindings differ in other factors however. NaCl added to Hb A with IHP causes an increased Bohr effect and an increased sensitivity of the cooperativity to pH. NaCl added to Hb A with ATP causes a reduction in the Bohr effect and a reduction in the sensitivity of the cooperativity to pH. This further demonstrates that IHP and ATP differ in their mechanism of interaction with murre Hb A. Overall the effect of NaCl seems to be to increase the affinity and reduce the cooperativity slightly.

Bindings with different concentrations of Tris HCl buffer showed a slight increase in affinity with an increase in the buffer concentration. This may be due to the increased Cl^- concentration. The increase in cooperativity with increase in buffer concentration can be explained as a result of increased ionic strength. Over the range of concentrations studied these results are in agreement with the results of Rossi-Fanelli et al., (1961) for human hemoglobin.

Functionally murre blood consists of two very different components. The major component (Hb A) is a high

affinity pigment which is very sensitive to the effects of IHP and ATP in reducing that affinity. The fairly high heme-heme interaction of stripped Hb A is reduced by the presence of IHP. Hb A with either IHP or ATP present shows an increase in affinity when NaCl is added. The minor component (Hb B) shows a low affinity and is relatively insensitive to IHP. The heme-heme interaction of stripped Hb B is relatively low and essentially unaffected by IHP. NaCl does increase the affinity of stripped Hb B. This suggests that the affinity of Hb B may be regulated mainly by changing chloride levels while the affinity of Hb A can be regulated by other environmental factors to meet the differing needs of the murre.

Diving mammals usually have a hemoglobin with a high affinity and a low Bohr effect. These properties along with bradycardia and vasoconstriction serve to reduce the oxygen flow to the tissues thus reserving the supply of oxygen for the vital organs. As shown by Lenfant et al., (1969) the Adélie penguin has a hemoglobin with similar properties. However, the penguin does not fly and its hemoglobin does not need to be adapted for flight. The rigors of flight demand a high delivery of oxygen to the tissues. To achieve this, flying birds need a hemoglobin with a high Bohr effect and a low affinity for oxygen. The murres, as well as other birds

which both dive and fly, need a compromise between these two conditions. The functional adaptations of the multiple components of murre hemoglobin may represent such a compromise. When the murre dives the major component Hb A would lose its oxygen relatively quickly due to its low affinity and high Bohr effect in the presence of allosteric modifiers. The value of Hb A as a storage molecule would be limited. However, the minor component, Hb B, has a somewhat higher affinity under these conditions and is less sensitive to environmental changes. It would tend to release its oxygen more gradually and thus meet the needs of the vital organs over a more extended period of time. With only 20% of the hemoglobin functioning as a fairly good storage molecule, one would not expect the murre to be capable of long duration dives such as certain marine mammals are capable of. In fact the maximum dive of the murre is around 3 minutes. This, however, is a fairly long dive among the birds, except for penguins which do not need a hemoglobin which is also adapted for flight.

When the diving murre reaches the surface its hemoglobin needs change dramatically. If the Alcidae have cardiac responses similar to the duck the heart rate increases considerably and the circulation is restored to the non-vital tissues (Cook et al., 1976). The pH

would drop due to lactic acid built up in those tissues (Catlett and Johnston, 1973). Hb A now functions well in that the low affinity and high Bohr effect allow rapid delivery of oxygen to the tissues. During the short period between dives the murre must deliver enough oxygen to the tissues to repay its oxygen debt and replace its stored oxygen for the next dive.

Birds which dive and fly usually show certain physical adaptations to allow both activities. They generally have relatively small wings and a heavy wing loading. This facilitates diving but requires a constant rapid wingbeat for flying. The murre is no exception and is characterized by very direct and rapid flight. The muscular activity necessary for this type of flight would require a high oxygen delivery hemoglobin such as Hb A. While Hb B would not be as effective as Hb A, it would probably still function to some degree under these conditions.

The respiratory function of the hemoglobin of diving birds then represents a system of compromises. If the bird is going to fly it needs a high delivery hemoglobin which is sensitive to environmental factors such as Hb A. Long dives such as those of marine mammals and penguins require a much greater storage capacity than a minor component such as Hb B can provide. However, the murre

can reach a compromise by having a multiple hemoglobin system. There is enough of an increase in storage capacity due to Hb B to extend diving times to three minutes. This apparently is enough to make the murre a successful marine predator. Yet there is sufficient oxygen delivery due to Hb A to meet the demands of flight. It appears that flying ability and dive times greater than 3-5 minutes are incompatible. One reason, among others, is the limited ability of the hemoglobin to meet the demands of both activities.

There are three basic directions for additional research which may further clarify the function of the minor component of avian hemoglobins. First, the structural aspects of the minor component of murre and other avian hemoglobins should be examined. The structure of a hemoglobin which is not strongly affected by organic phosphates may help clarify the mechanism of action of these modifiers. Also the fact that Hb B did not dissolve well in 10 M urea would be of interest structurally. Secondly, the oxygen affinity of the two components under more physiological conditions should be examined. This may include the determination of organic phosphate levels in murre red blood cells. Thirdly, comparative oxygen affinity studies on the individual components of other non-domestic birds could be undertaken to determine

any functional adaptations they may exhibit.

Table 1. Characteristics of the oxygen dissociation curves. Bohr effect, P_{50} at pH 7.4, Hill coefficient "h" at pH 7.4 and slope "h" vs pH for murre hemoglobin components under various conditions. All determinations in 0.01 M Tris HCl at 20° C.

TABLE I

SOLUTION	$\phi = \frac{\Delta \text{ LOG P50}}{\Delta \text{ pH}}$	P50 (pH = 7.4)	"h" (pH = 7.4)	SLOPE "h" vs pH
Hb A STRIPPED	0 (7.2-7.6)	2.0	2.9	———
Hb A with NaCl	-0.40	1.75	2.75	0
Hb A with IHP	-0.20	20.9	1.76	0.67
Hb A with NaCl and IHP	-0.50	12.6	1.45	0.87
Hb A with ATP	-0.60	21.9	2.7	0.63
Hb A with NaCl and ATP	-0.46	13.8	2.6	0.45
Hb B STRIPPED	-0.27	17.4	1.7	0.20
Hb B with IHP	0	15.1	1.65	0
Hb B with NaCl	-0.45	7.2	1.35	0.60

Figure 1. Scan of isoelectric focusing gel for murre whole COHb. Absorbance vs distance migrated. Also showing pH gradient of the gel.

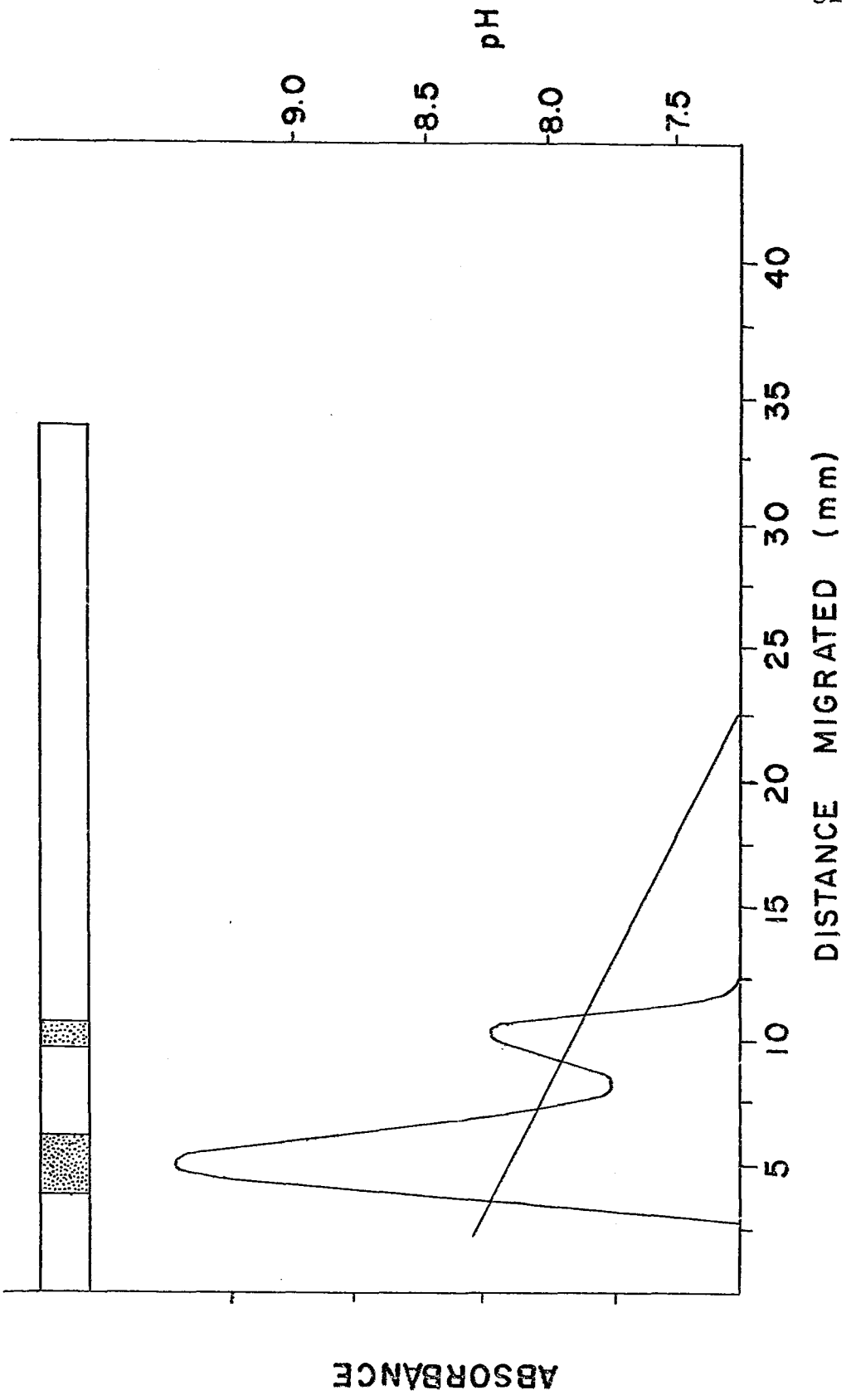

pH
9.0
8.5
8.0
7.5
5
10
15
20
25
30
35
40
DISTANCE MIGRATED (mm)
ABSORBANCE

FIGURE 1

Figure 2. Elution patterns of murre hemoglobin from DEAE cellulose ion exchange column. Buffer is 0.01 M Tris HCl pH 8.0. Second peak eluted with this buffer plus 0.01 M NaCl.

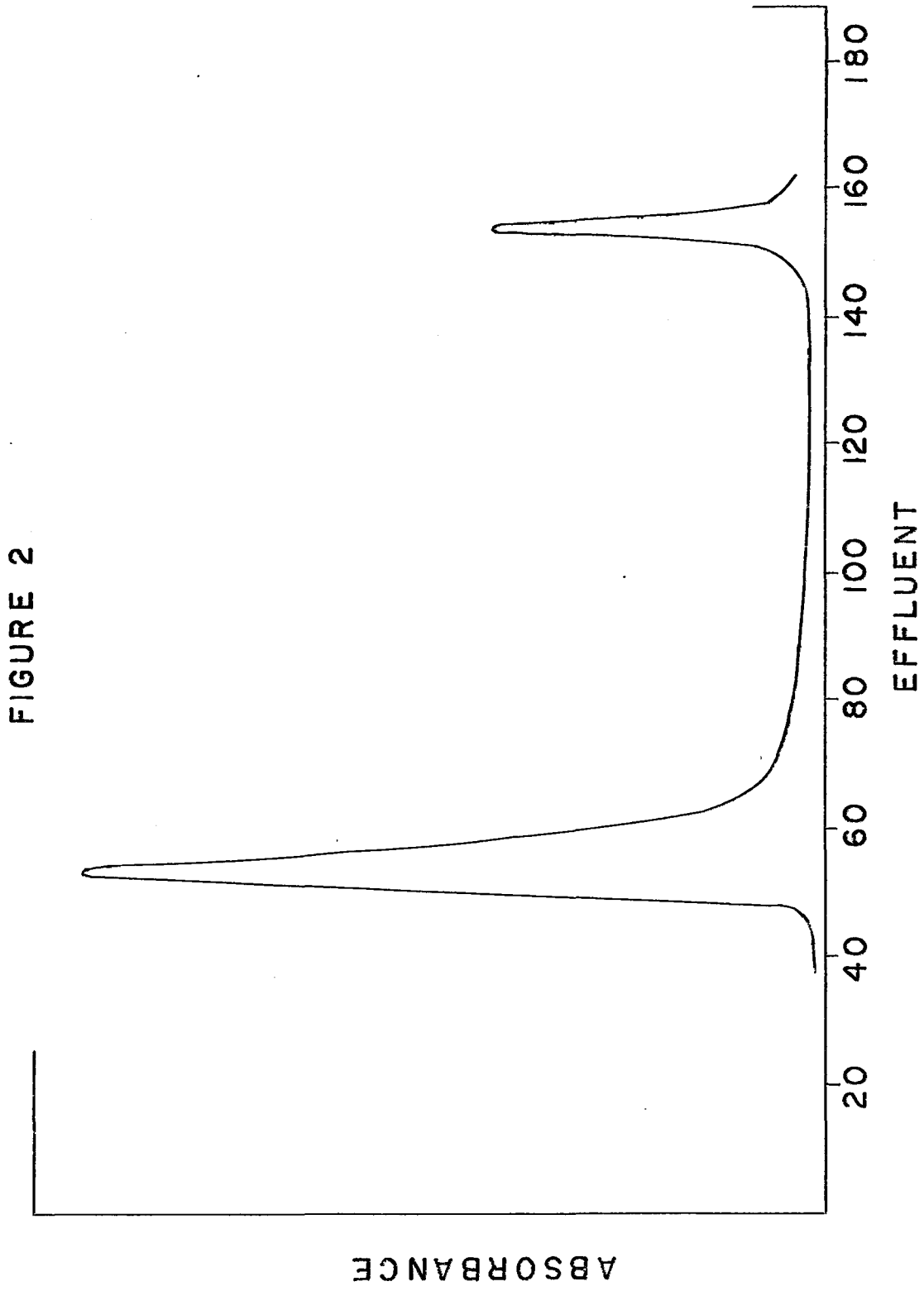


FIGURE 2

Figure 3. Electrophoretic pattern of murre globins on 6.25 M urea gel electrophoresis. Gel 1 shows Hb A, gel 2 shows Hb B, and gel 3 shows Hb A and Hb B together.

FIGURE 3

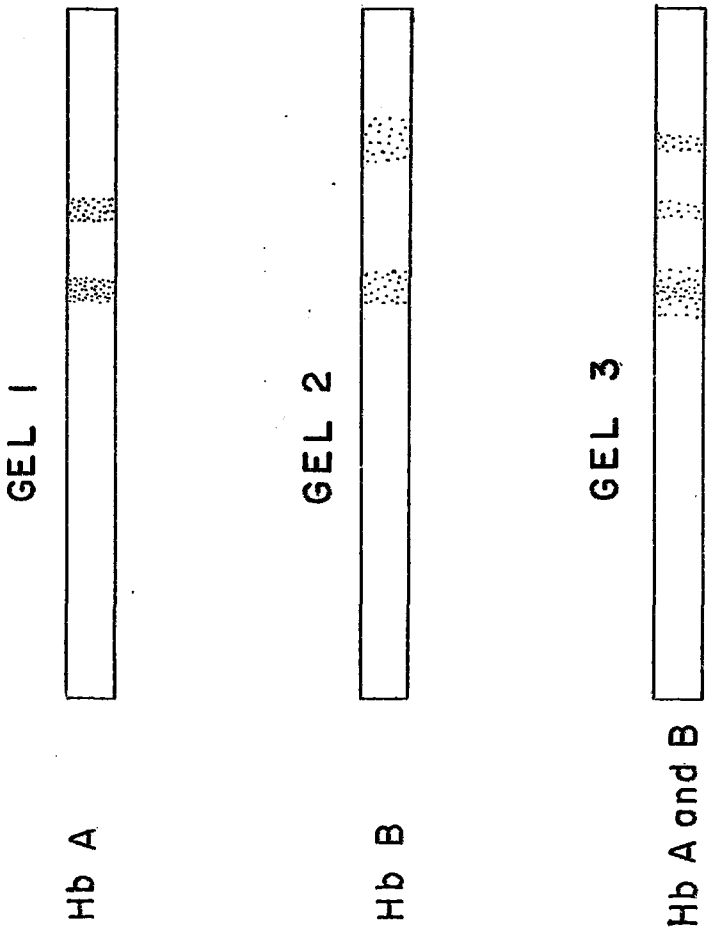


Figure 4. Oxygen affinity of stripped murre Hb A. Log P_{50} vs pH. Temperature is 20° C. Buffer is 0.01 M Tris HCl.

FIGURE 4

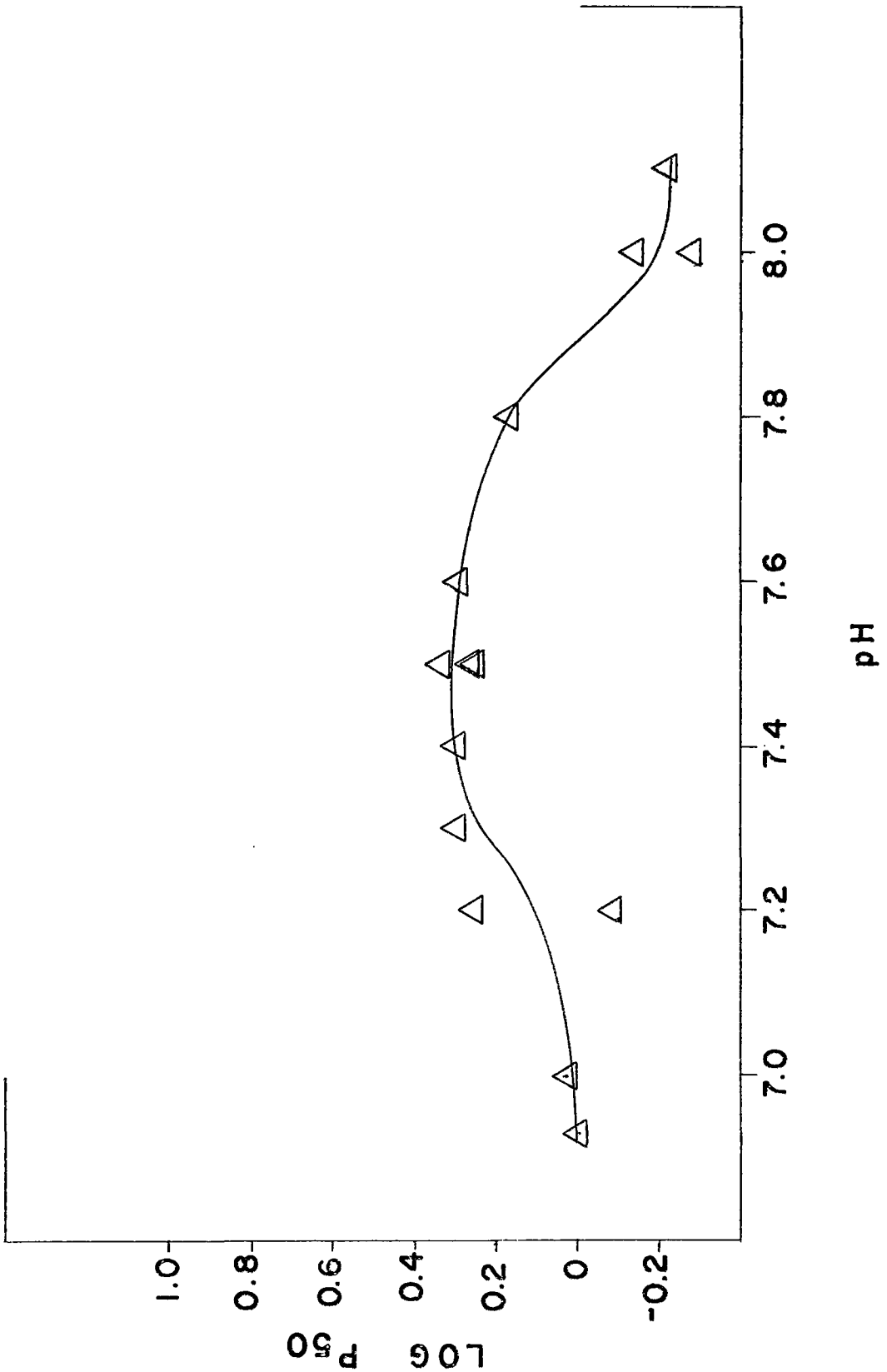


Figure 5. Cooperativity of stripped murre Hb A. Hill coefficient "h" vs pH. Temperature 20° C. Buffer is 0.01 M Tris HCl.

FIGURE 5

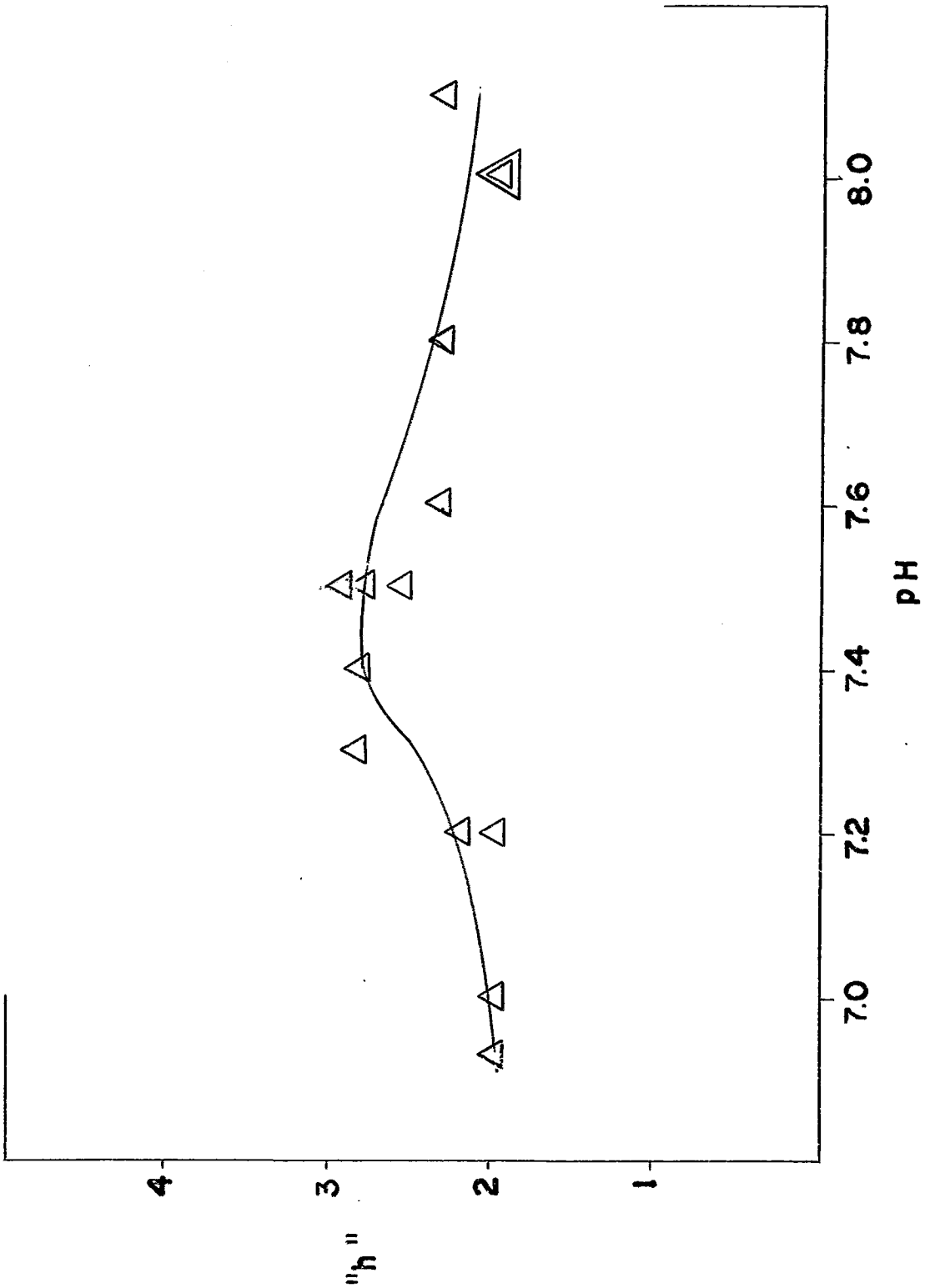


Figure 6. Oxygen affinity of stripped murre Hb A with 0.1 M NaCl added. Log P_{50} vs pH. Temperature is 20° C. Buffer is 0.01 M Tris HCl.

FIGURE 6

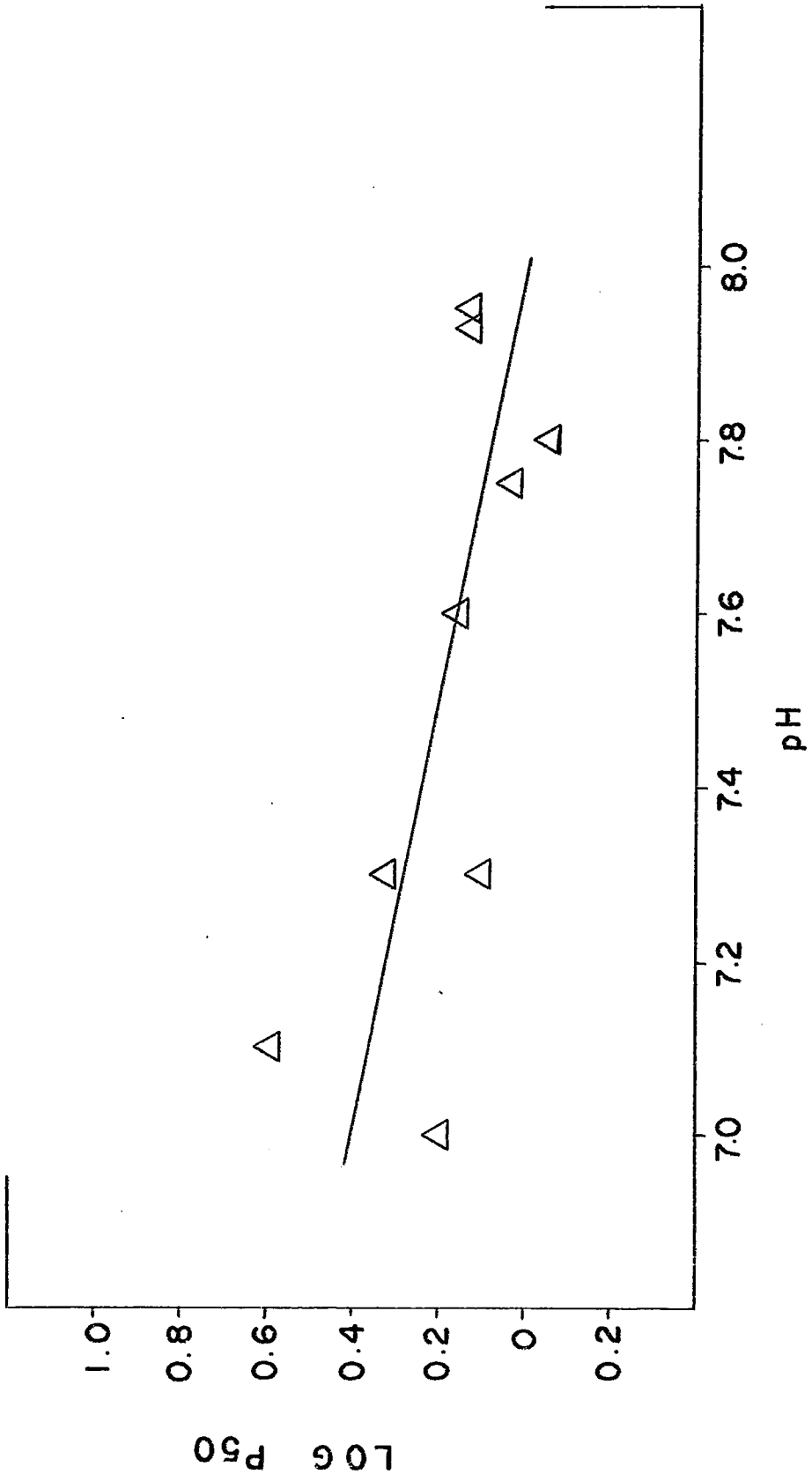


Figure 7. Cooperativity of stripped murre Hb A with 0.1 M NaCl added. Hill coefficient "h" vs pH. Temperature is 20° C. Buffer is 0.01 M Tris HCl.

FIGURE 7

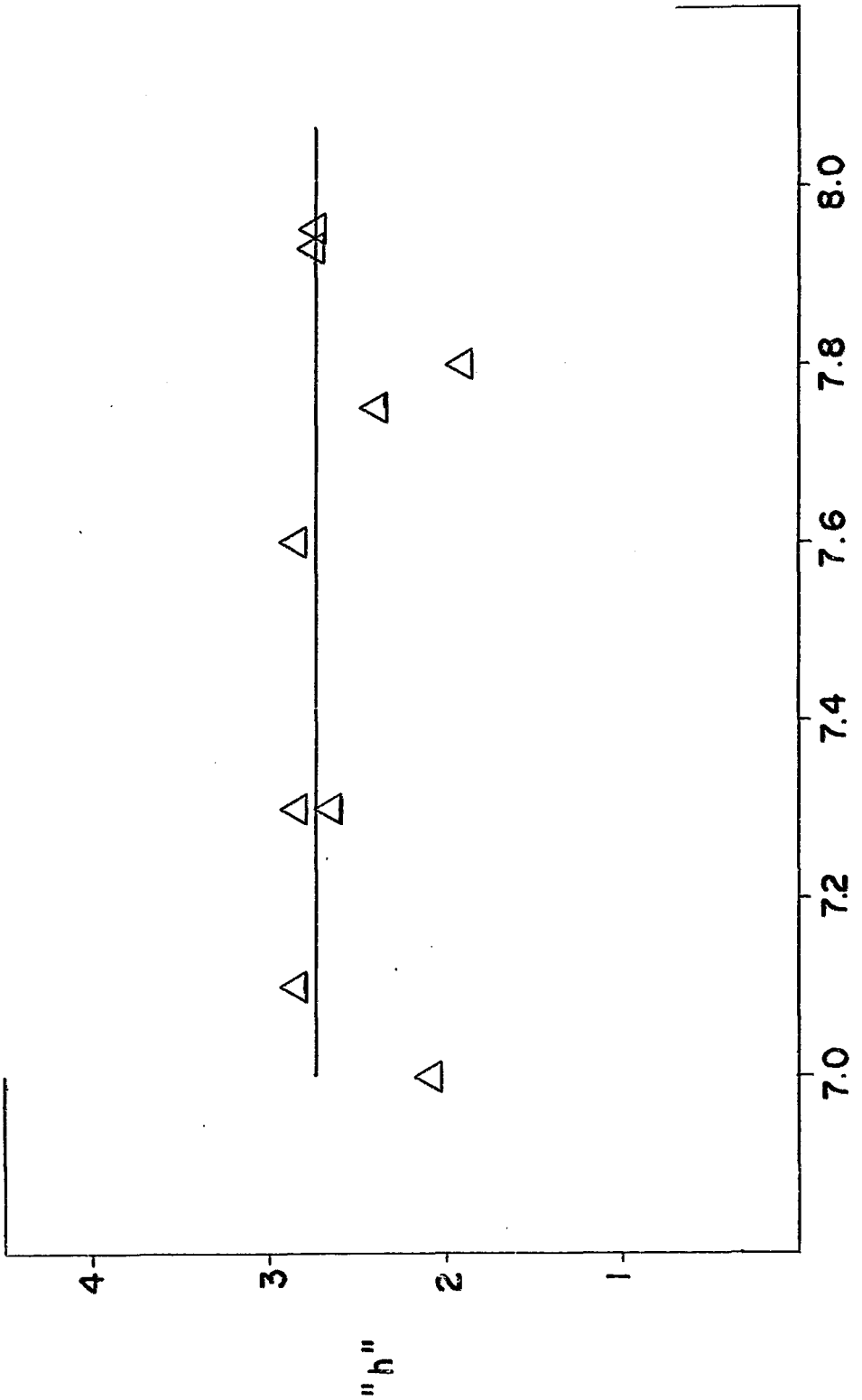


Figure 8. Oxygen affinity of stripped murre Hb A with 1 mM IHP added. Log P_{50} vs pH. Temperature is 20° C. Buffer is 0.01 M Tris HCl.

FIGURE 8

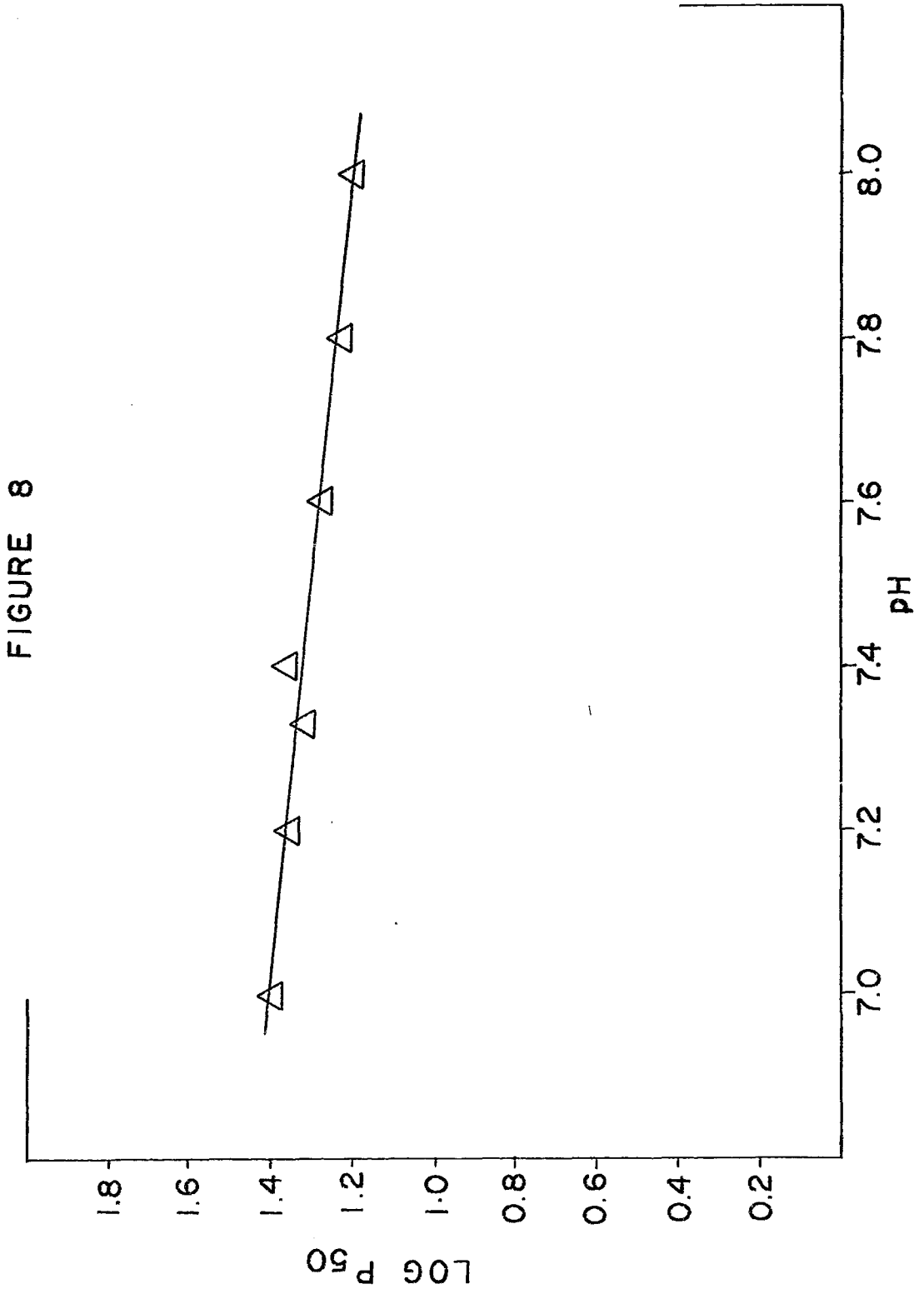


Figure 9. Cooperativity of stripped murre Hb A with 1 mM IHP added. Hill coefficient "h" vs pH. Temperature 20° C. Buffer is 0.01 M Tris HCl.

FIGURE 9

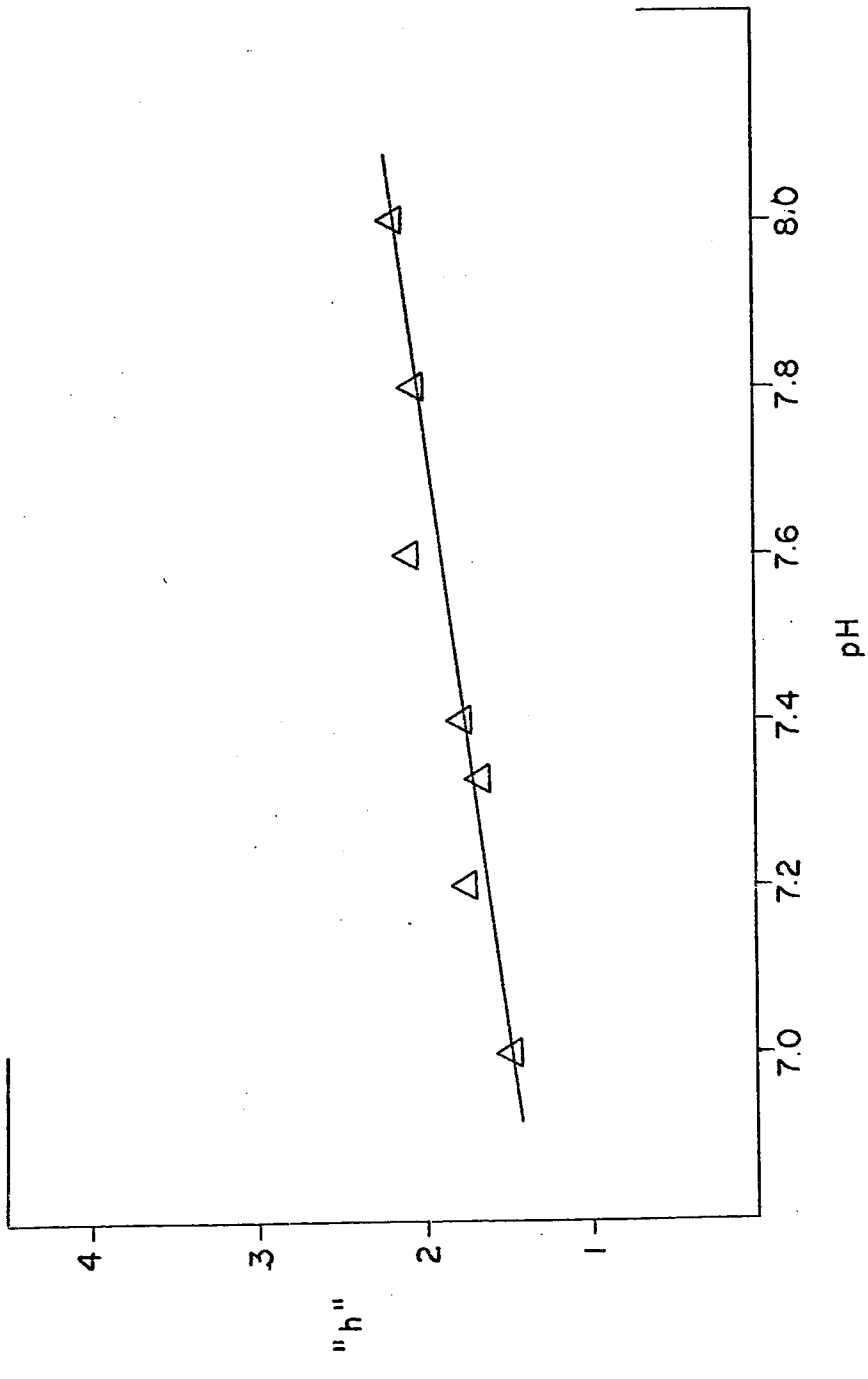


Figure 10. Oxygen affinity of stripped murre Hb A with 1 mM IHP and 0.1 M NaCl added. Log P_{50} vs pH. Temperature 20° C. Buffer is 0.01 M Tris HCl.

FIGURE 10

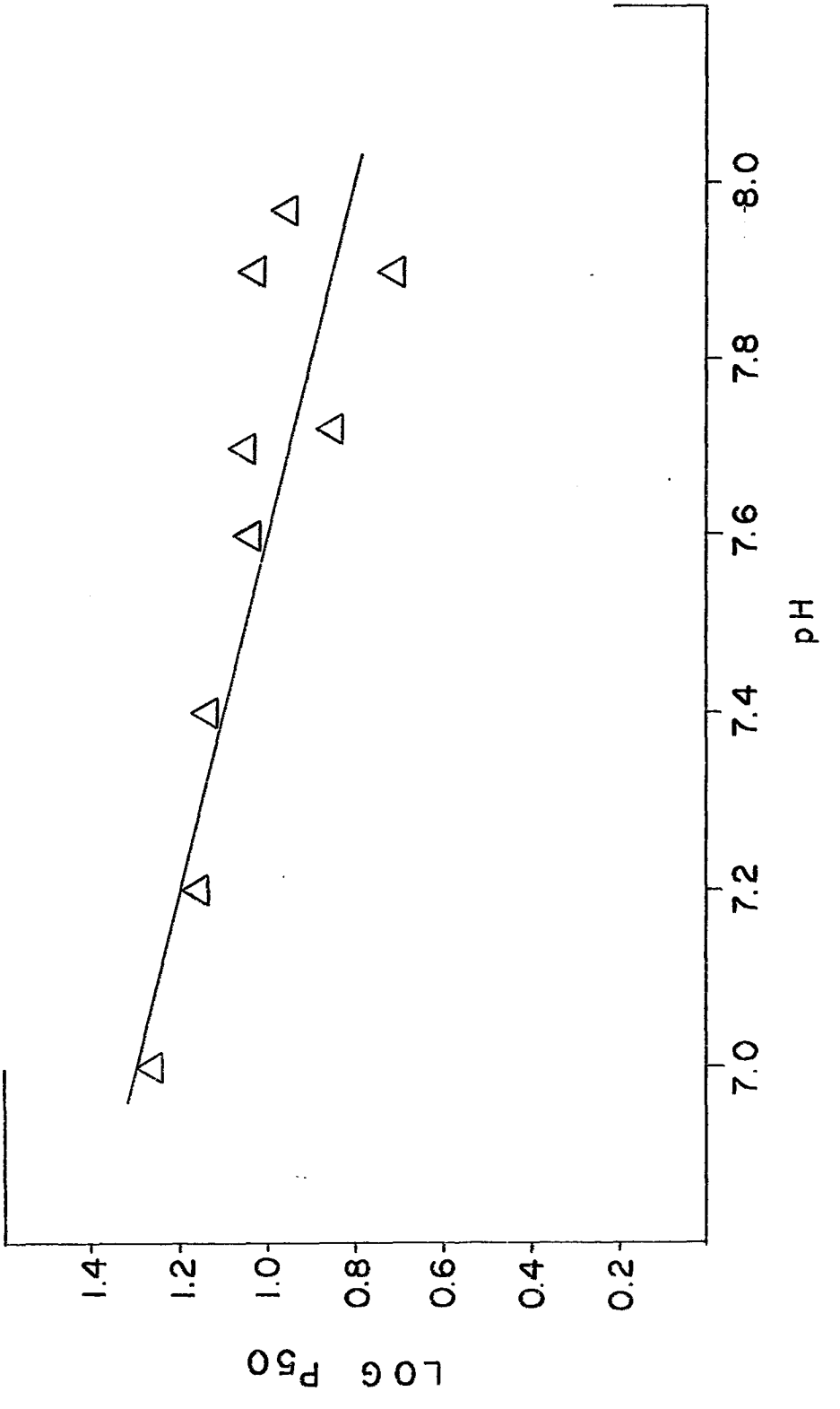


Figure 11. Comparison of the oxygen affinity of stripped murre Hb A (——), stripped murre Hb A with 0.1 M NaCl added (- - -), stripped murre Hb A with 1 mM IHP added (•••——), and stripped murre Hb A with 0.01 M NaCl and 1 mM IHP added (•••••). Log P_{50} vs Ph. Temperature is 20° C. Buffer is 0.01 M Tris HCl.

FIGURE II

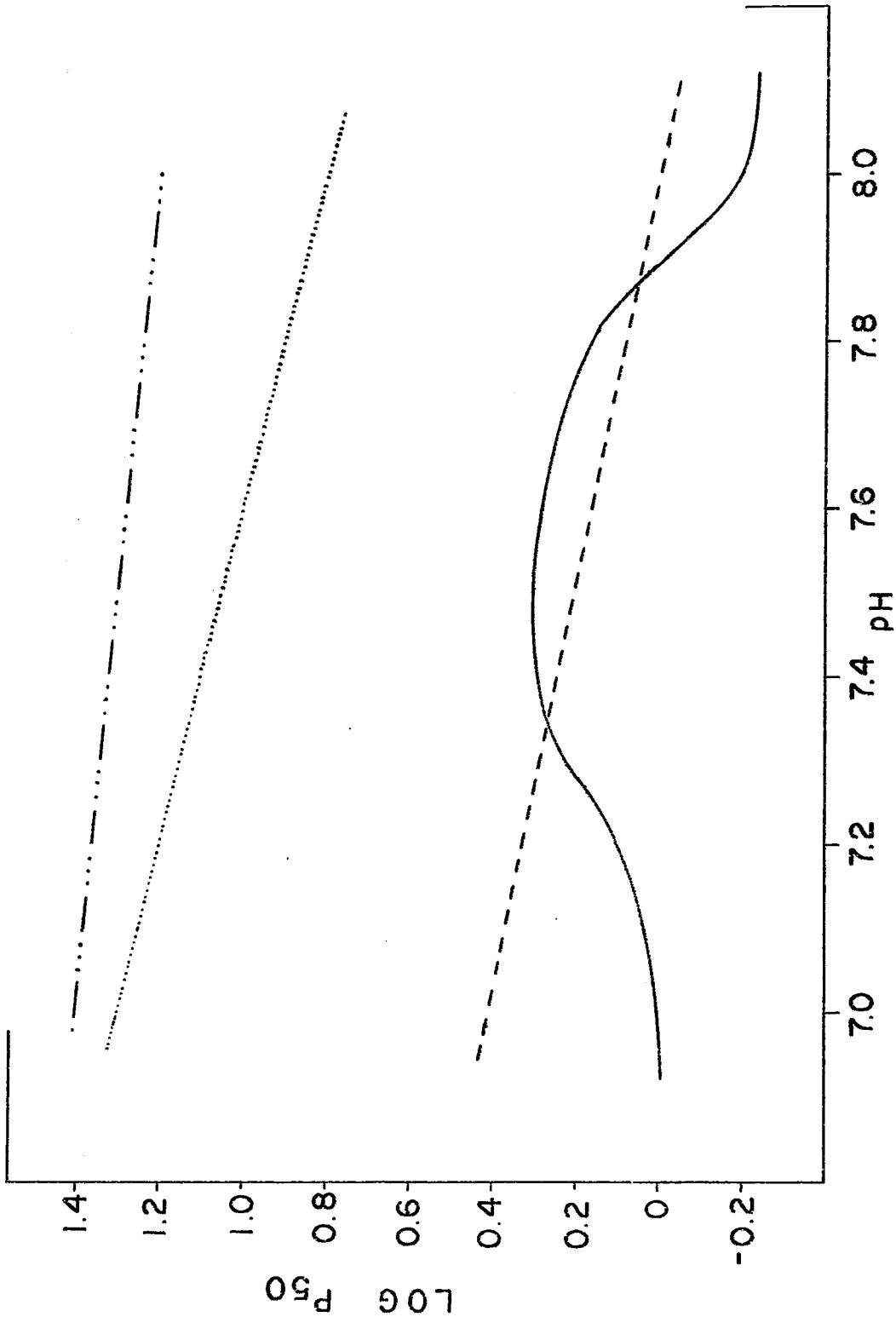


Figure 12. Cooperativity of stripped murre Hb A with 0.1 M NaCl and 1 mM IHP added. Hill coefficient "h" vs pH. Temperature 20° C. Buffer is 0.01 M Tris HCl.

FIGURE 12

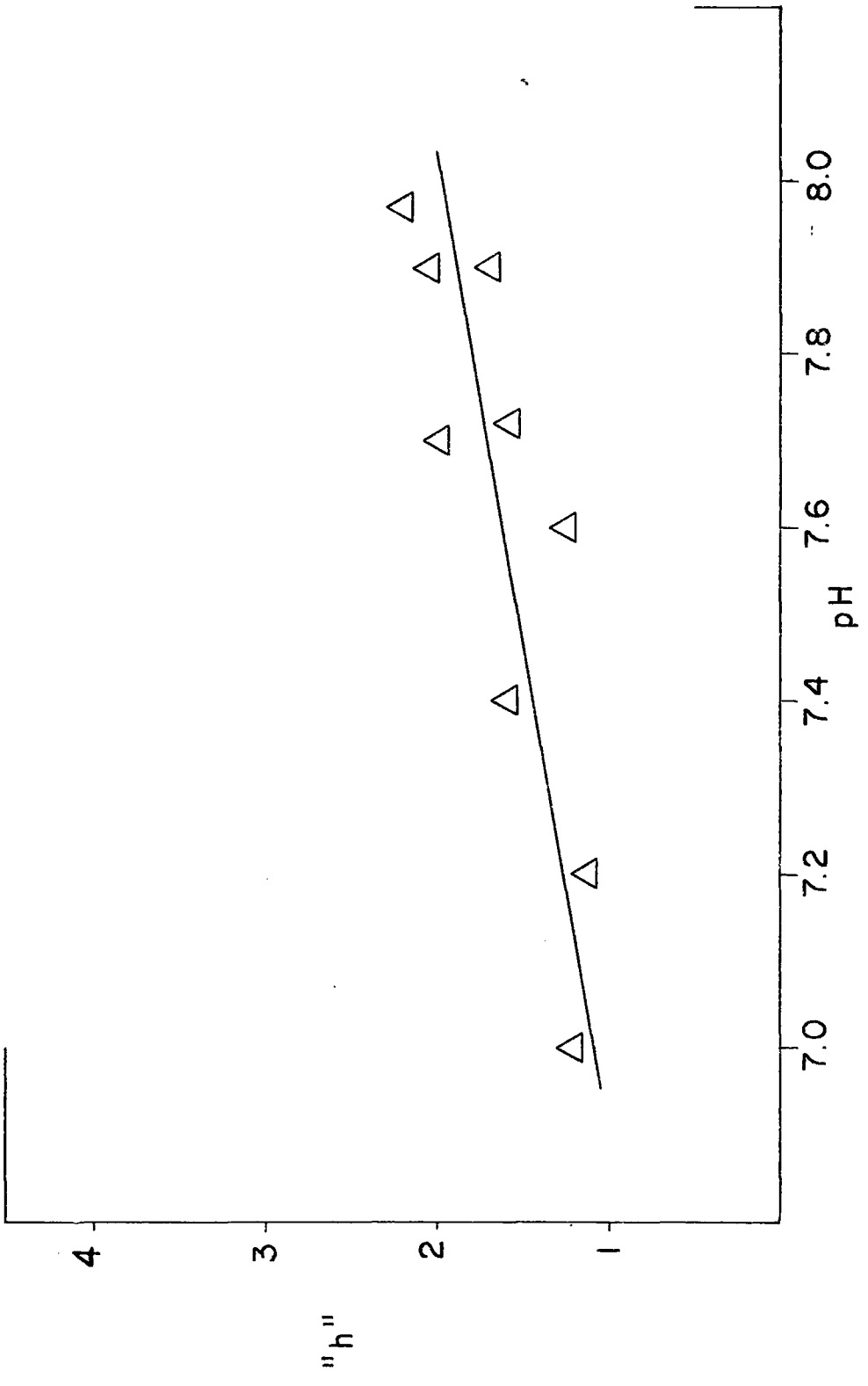


Figure 13. Oxygen affinity of stripped murre Hb A with 1 mM ATP. Log P_{50} vs pH. Temperature 20° C. Buffer is 0.01 M Tris HCl.

FIGURE 13

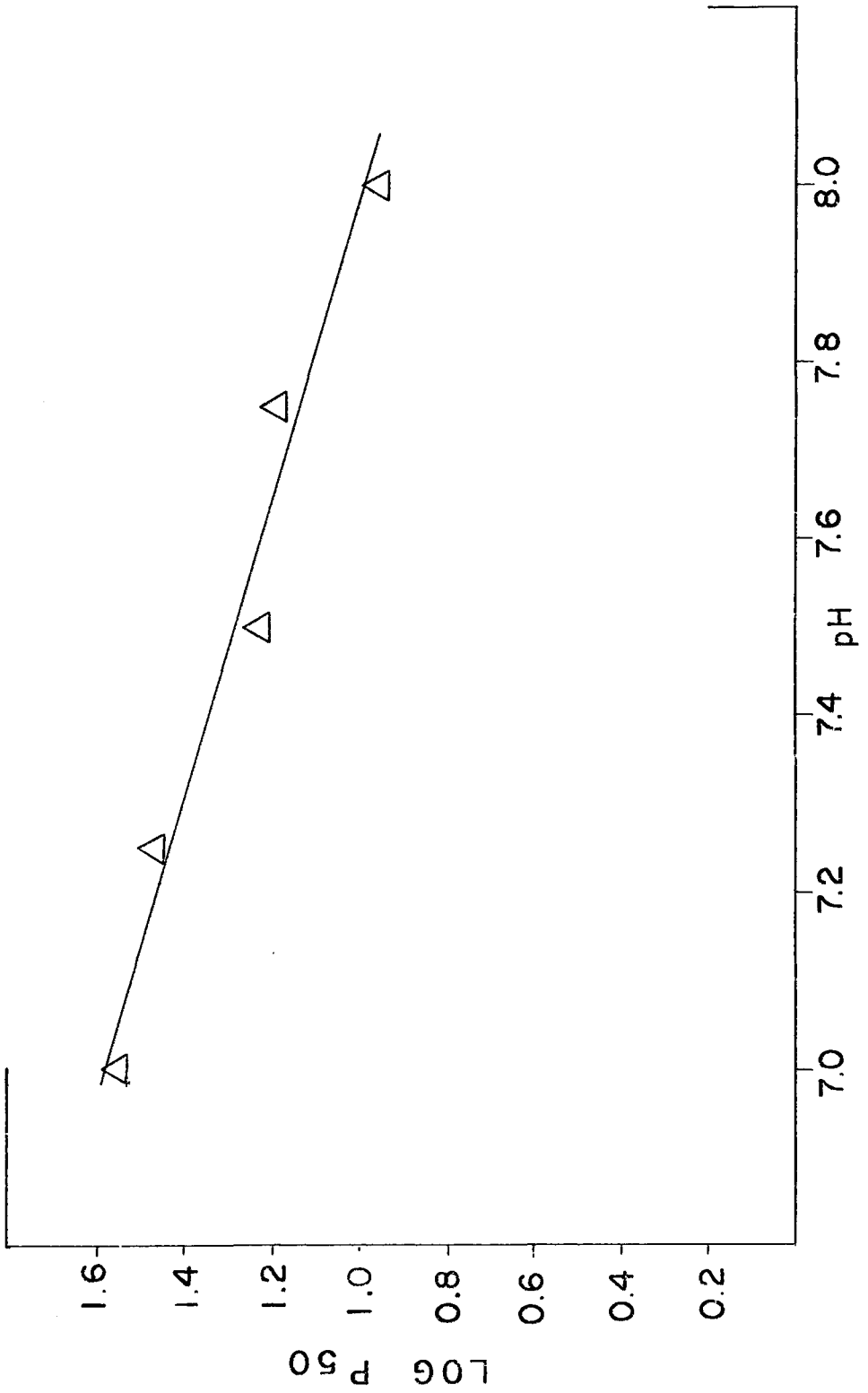


Figure 14. Cooperativity of stripped murre Hb A with 1 mM ATP added. Hill coefficient "h" vs pH. Temperature is 20° C. Buffer is 0.01 M Tris HCl.

FIGURE 14

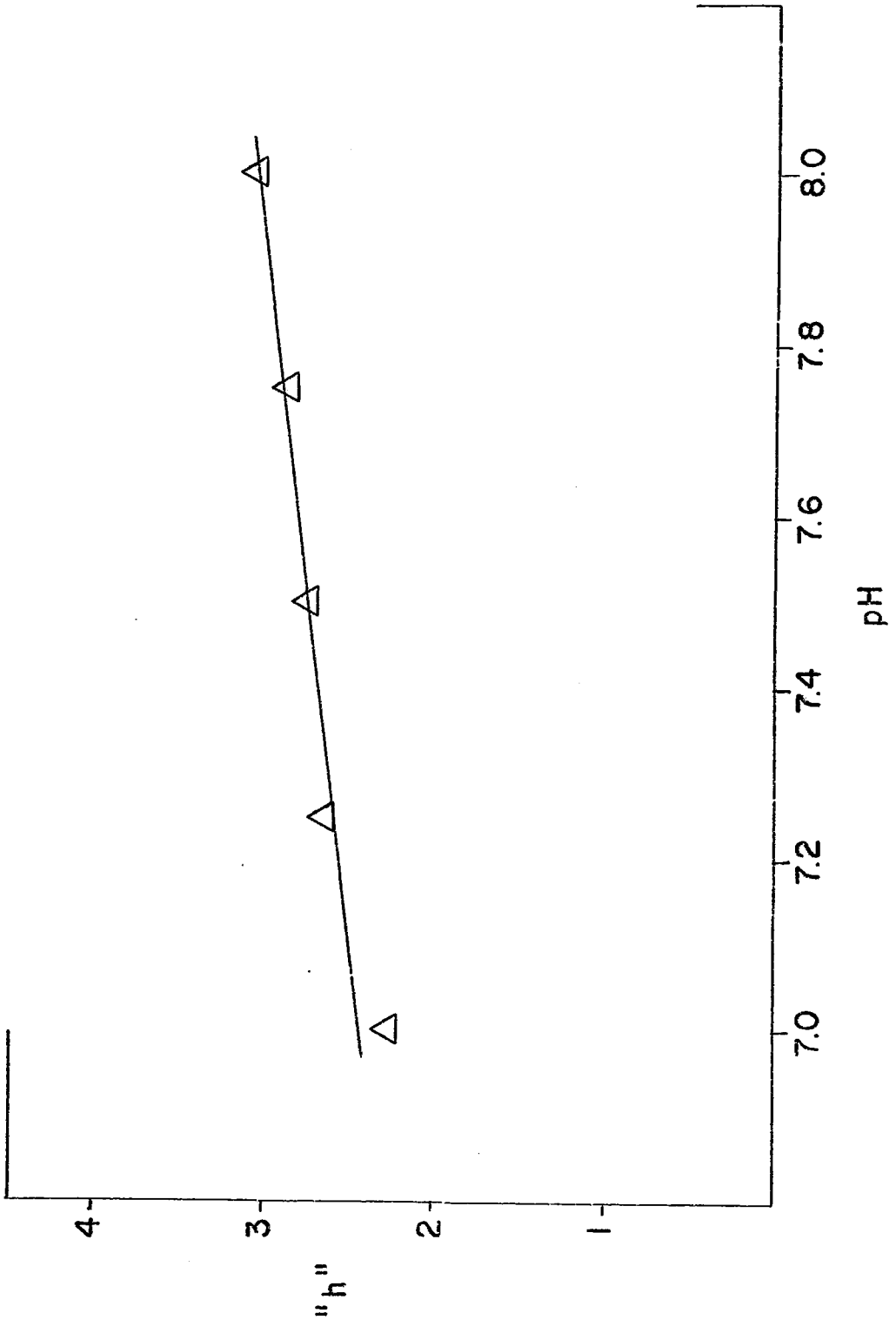


Figure 15. Oxygen affinity of stripped murre Hb A with 1 mM ATP and 0.1 M NaCl added. Log P_{50} vs pH. Temperature is 20° C. Buffer is 0.01 M Tris HCl.

FIGURE 15

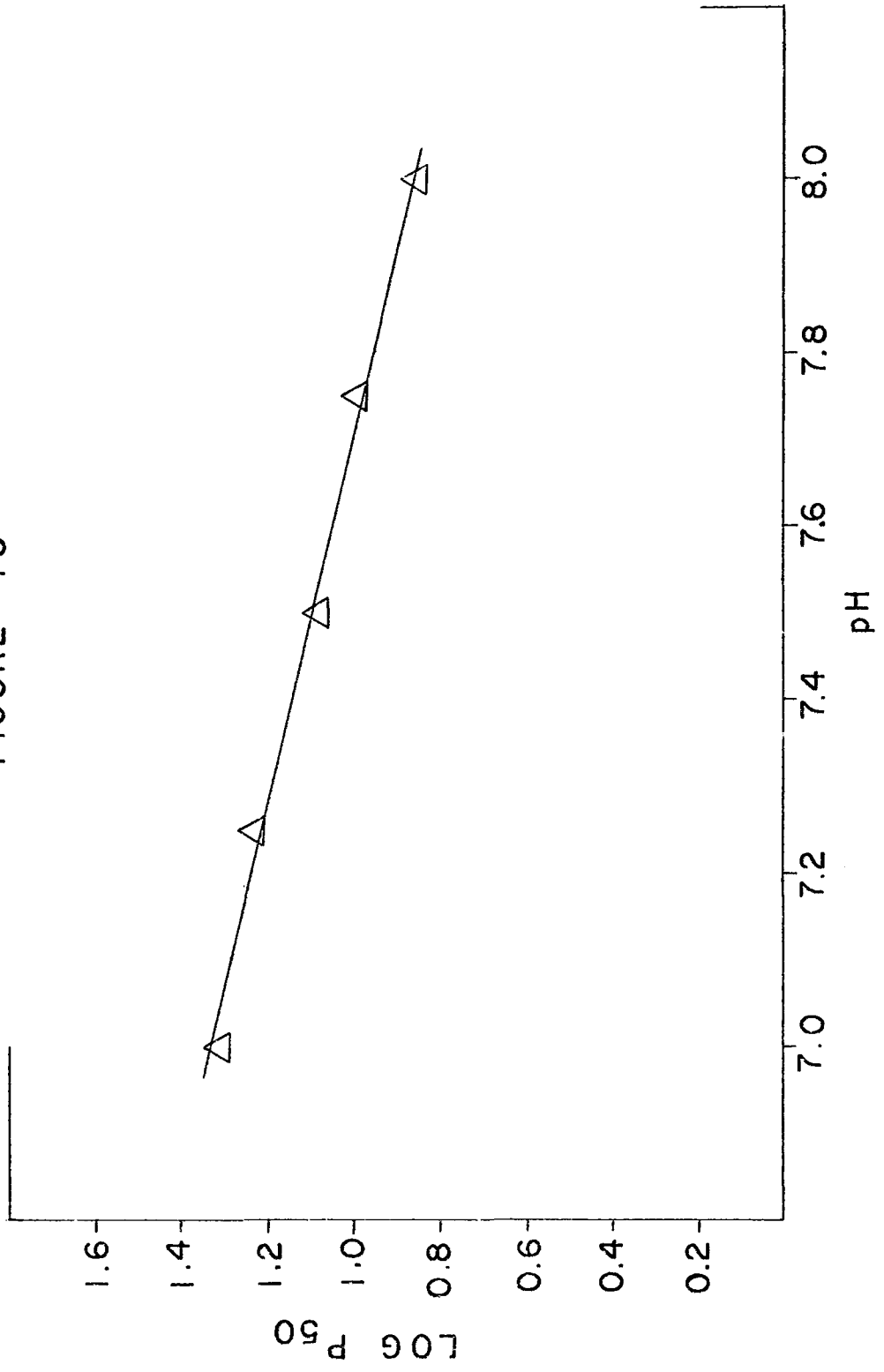


Figure 16. Cooperativity of stripped murre Hb A with 1 mM ATP and 0.1 M NaCl added. Hill coefficient "h" vs pH. Temperature is 20° C. Buffer is 0.01 M Tris HCl.

FIGURE 16

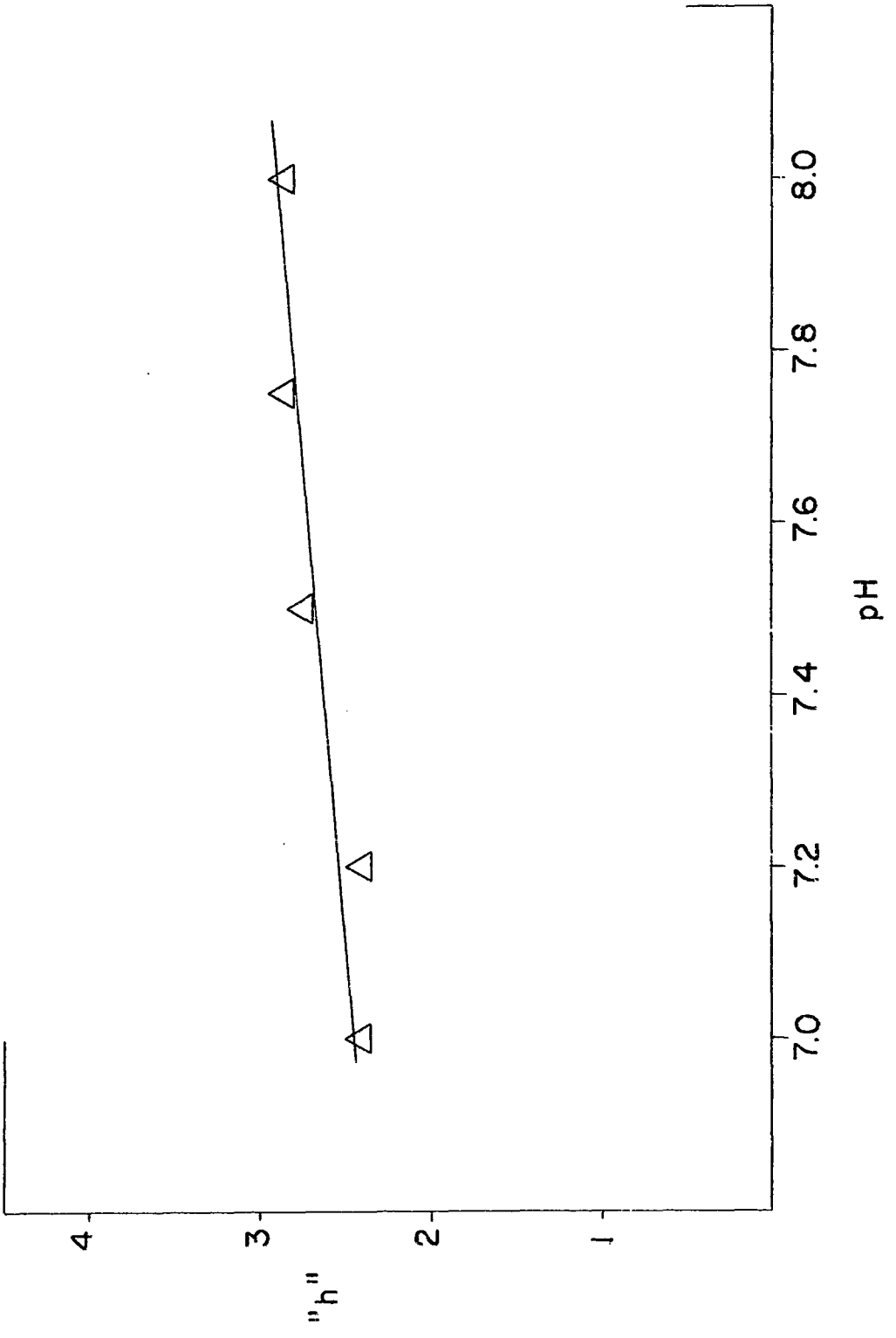


Figure 17. Oxygen affinity of stripped murre Hb B. Log P_{50} vs pH. Temperature is 20° C. Buffer is 0.01 M Tris HCl.

FIGURE 17

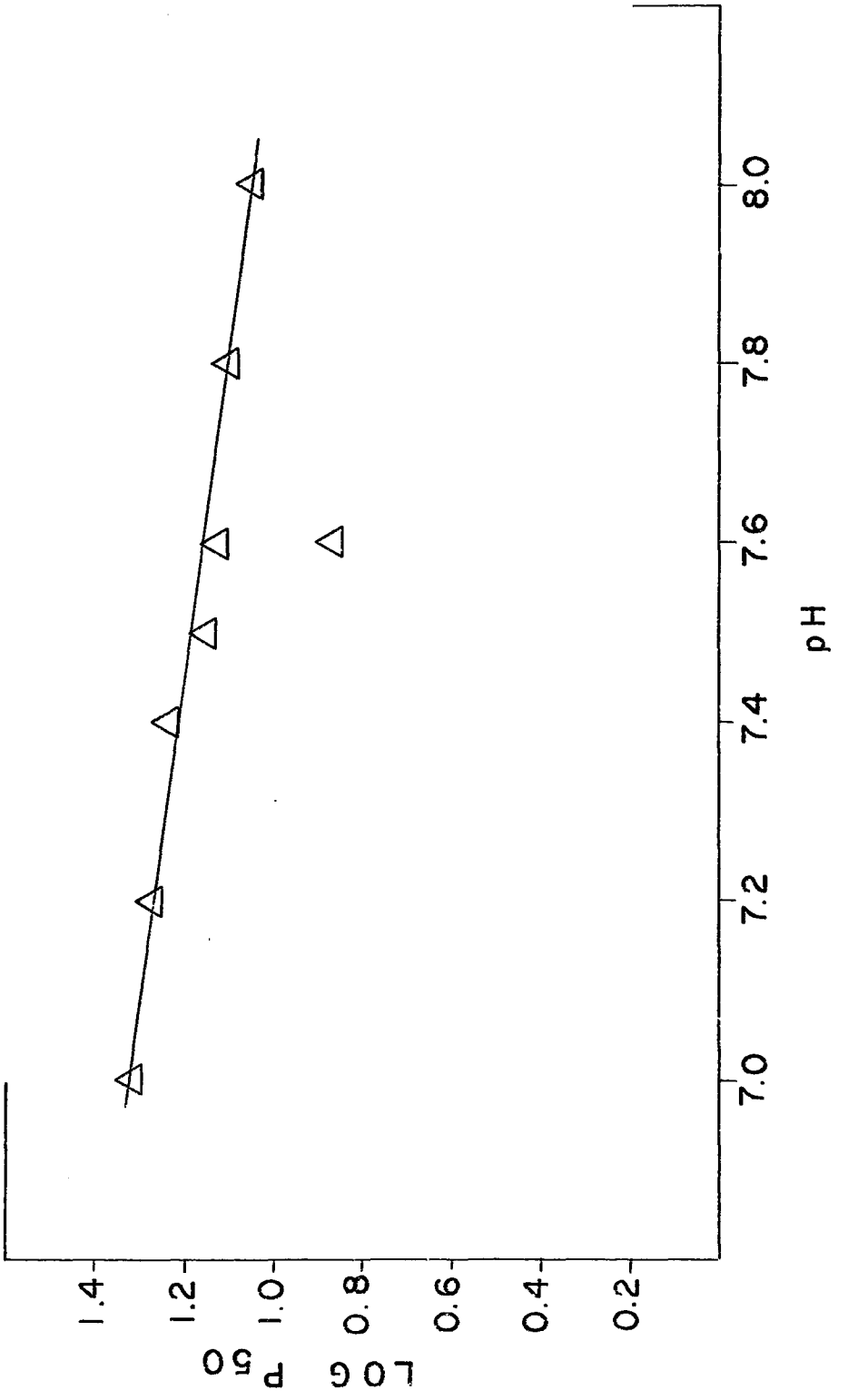


Figure 18. Cooperativity of stripped murre Hb B. Hill coefficient "h" vs pH. Temperature is 20° C. Buffer is 0.01 M Tris HCl.

FIGURE 18

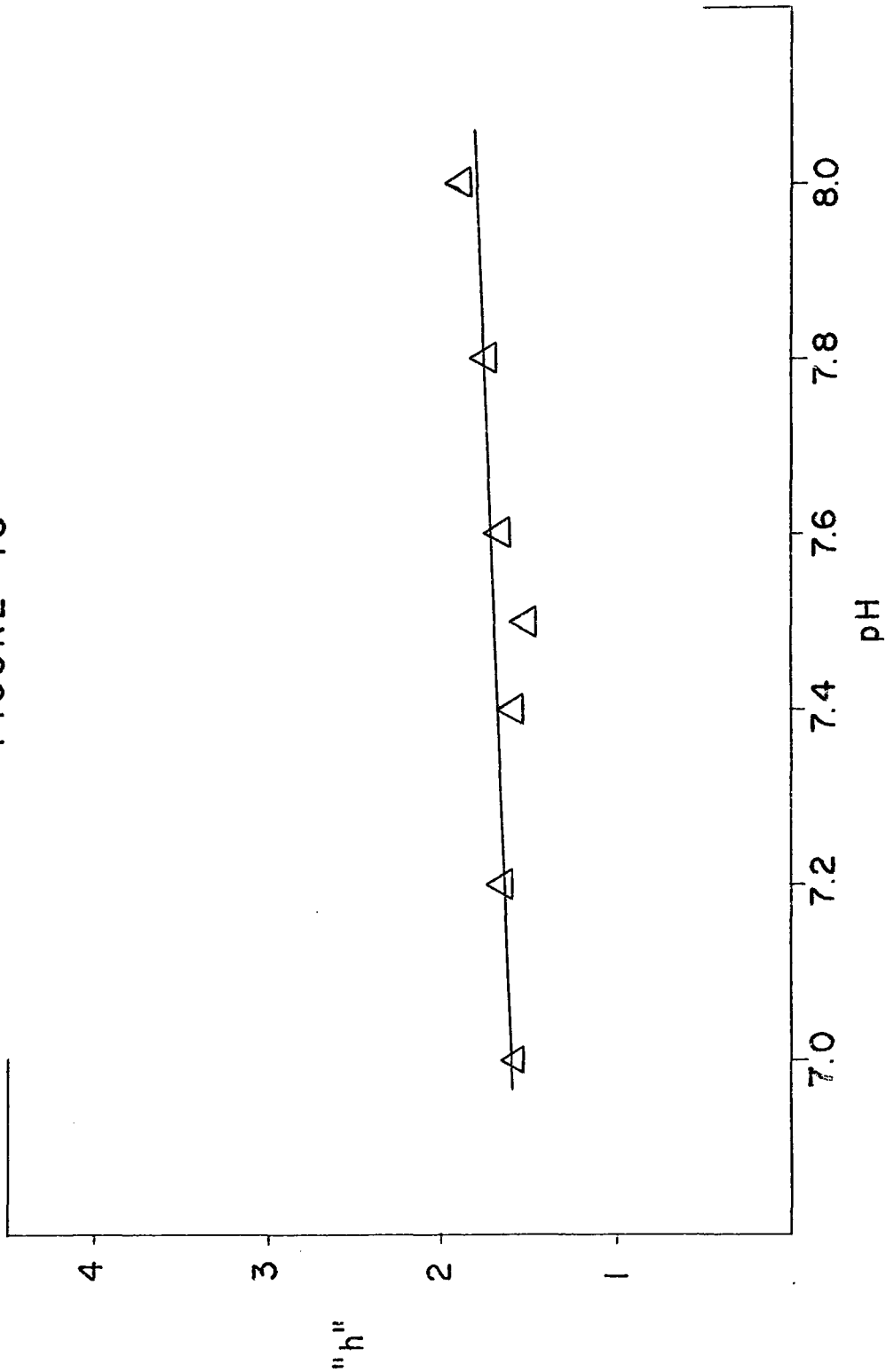


Figure 19. Oxygen affinity of stripped murre Hb B with 0.1 M NaCl added. Log P_{50} vs pH. Temperature is 20° C. Buffer is 0.01 M Tris HCl.

FIGURE 19

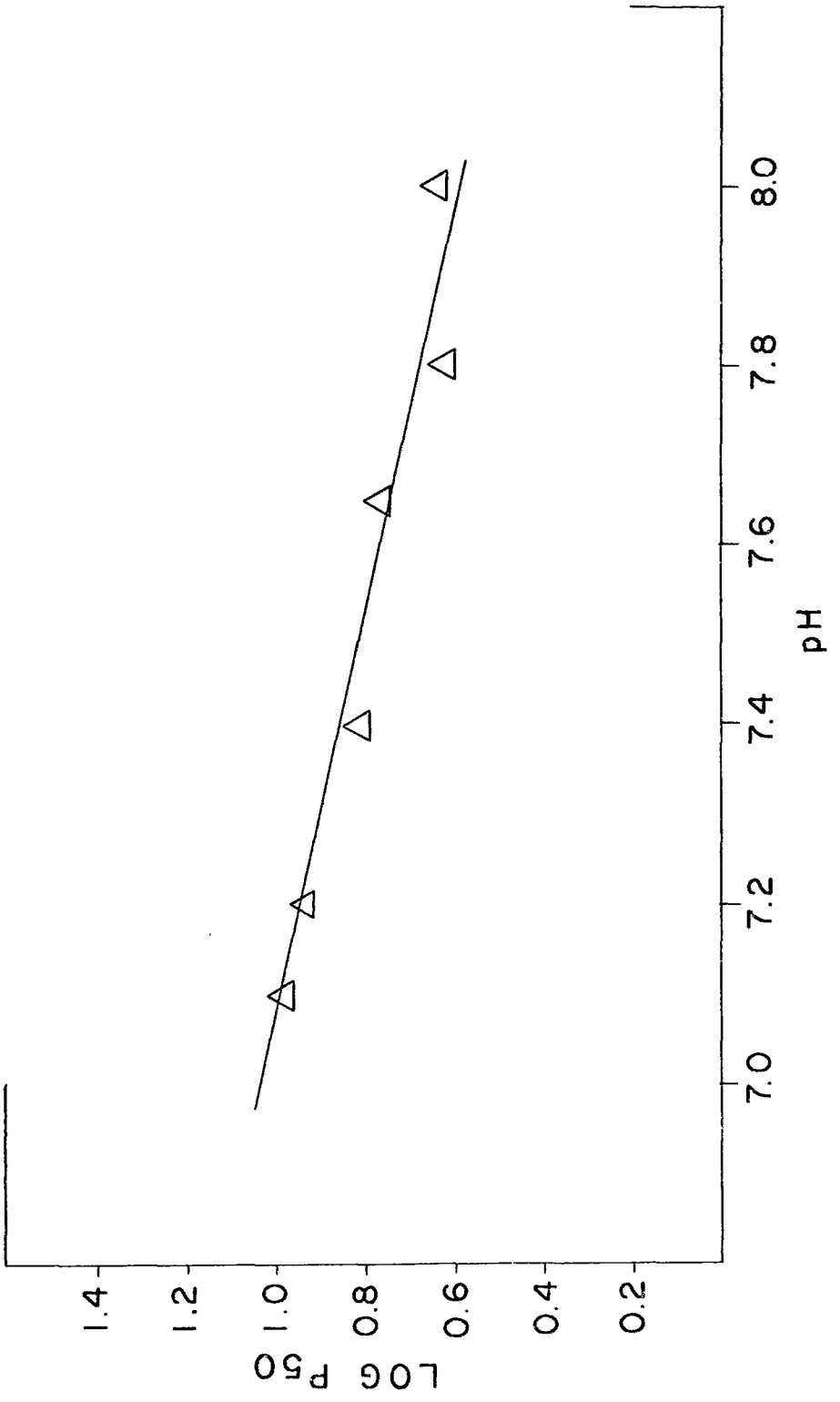


Figure 20. Cooperativity of stripped murre Hb B with 0.1 M NaCl added. Hill coefficient "h" vs pH. Temperature is 20° C. Buffer is 0.01 M Tris HCl.

FIGURE 20

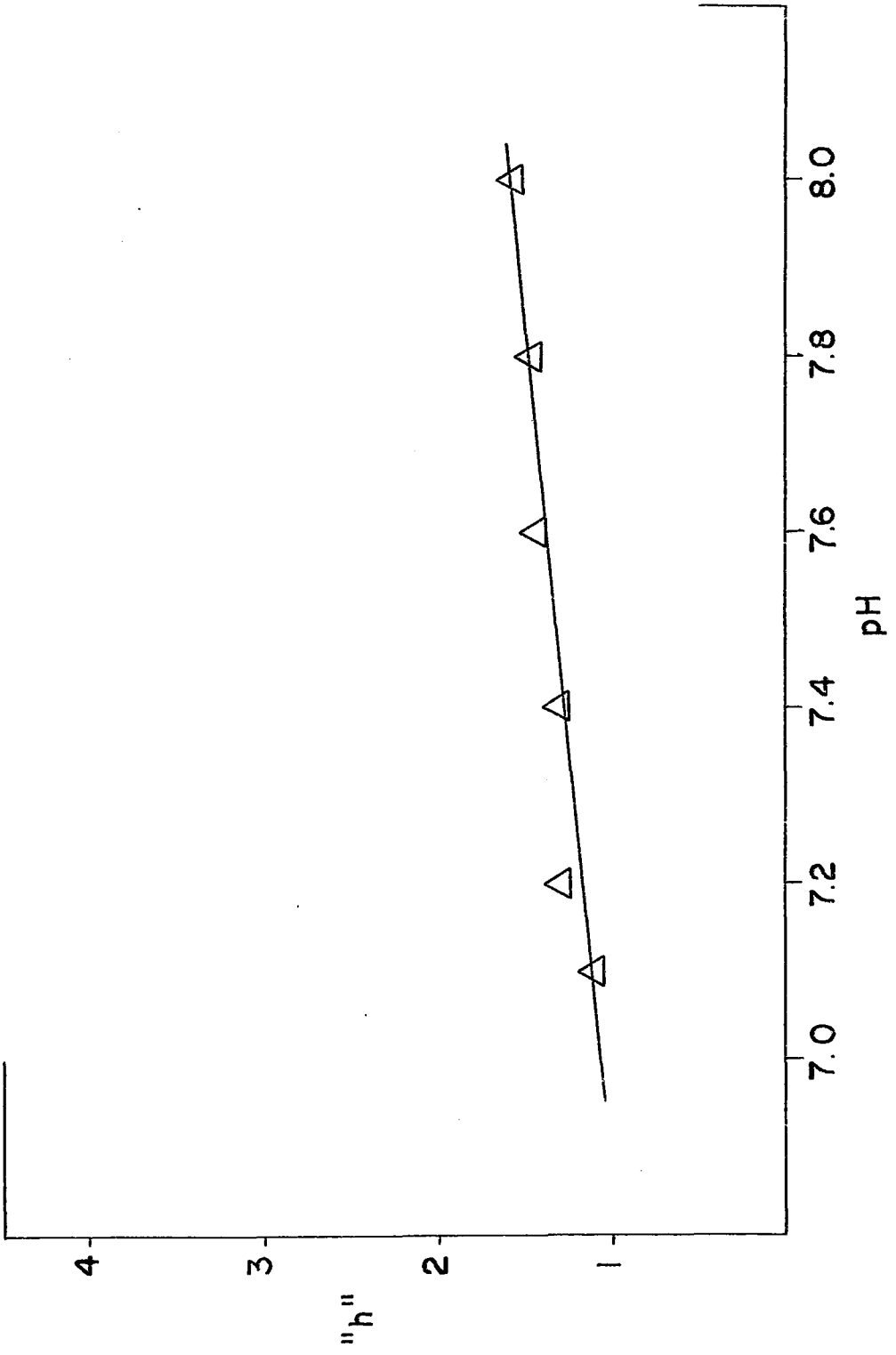


Figure 21. Oxygen affinity of stripped murre Hb B with 1 mM IHP added. Log P_{50} vs pH. Temperature is 20° C. Buffer is 0.01 M Tris HCl.

FIGURE 21

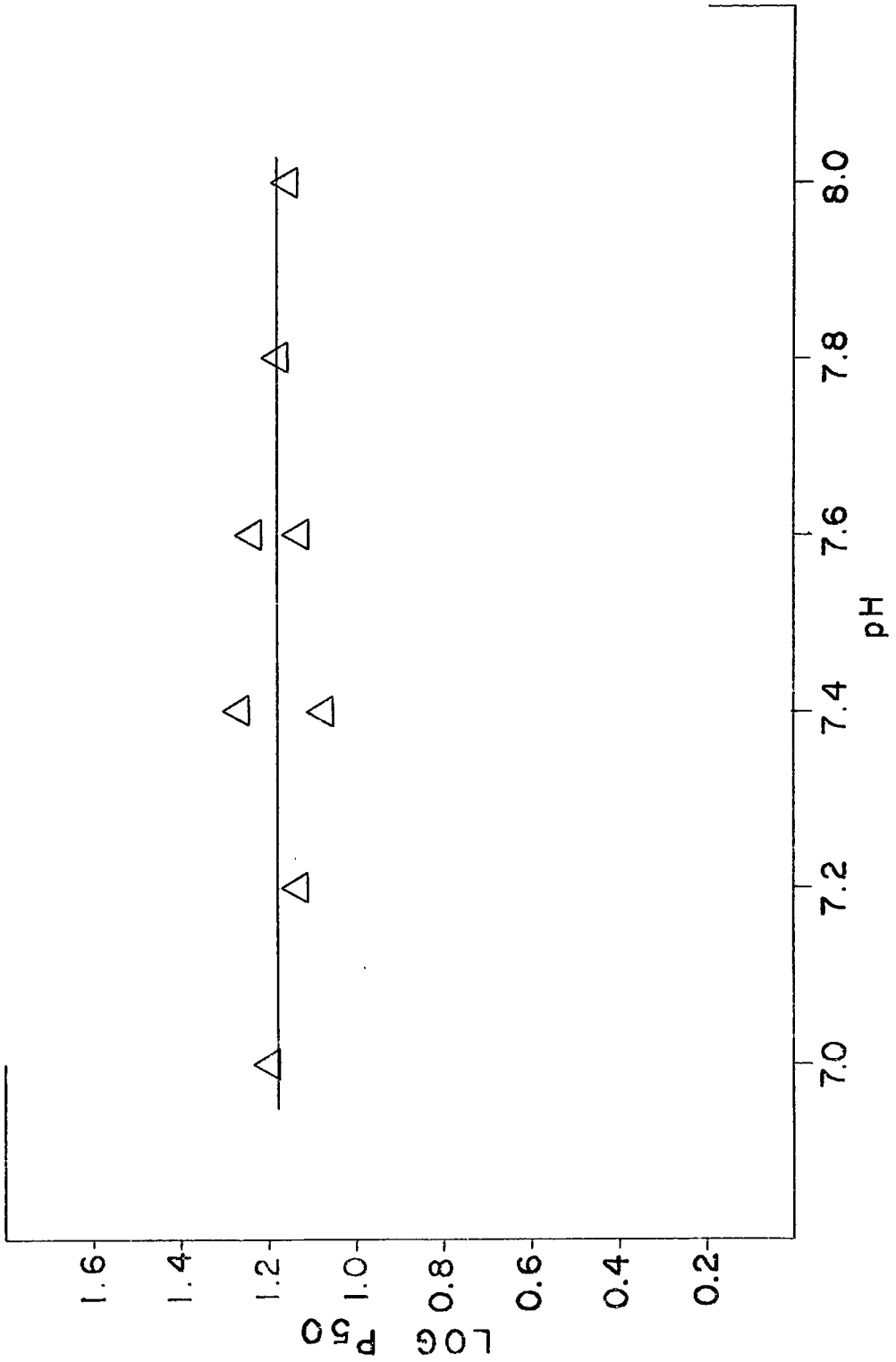


Figure 22. Cooperativity of stripped murre Hb B with 1 mM IHP added. Hill coefficient "h" vs pH. Temperature is 20° C. Buffer is 0.01 M Tris HCl.

FIGURE 22

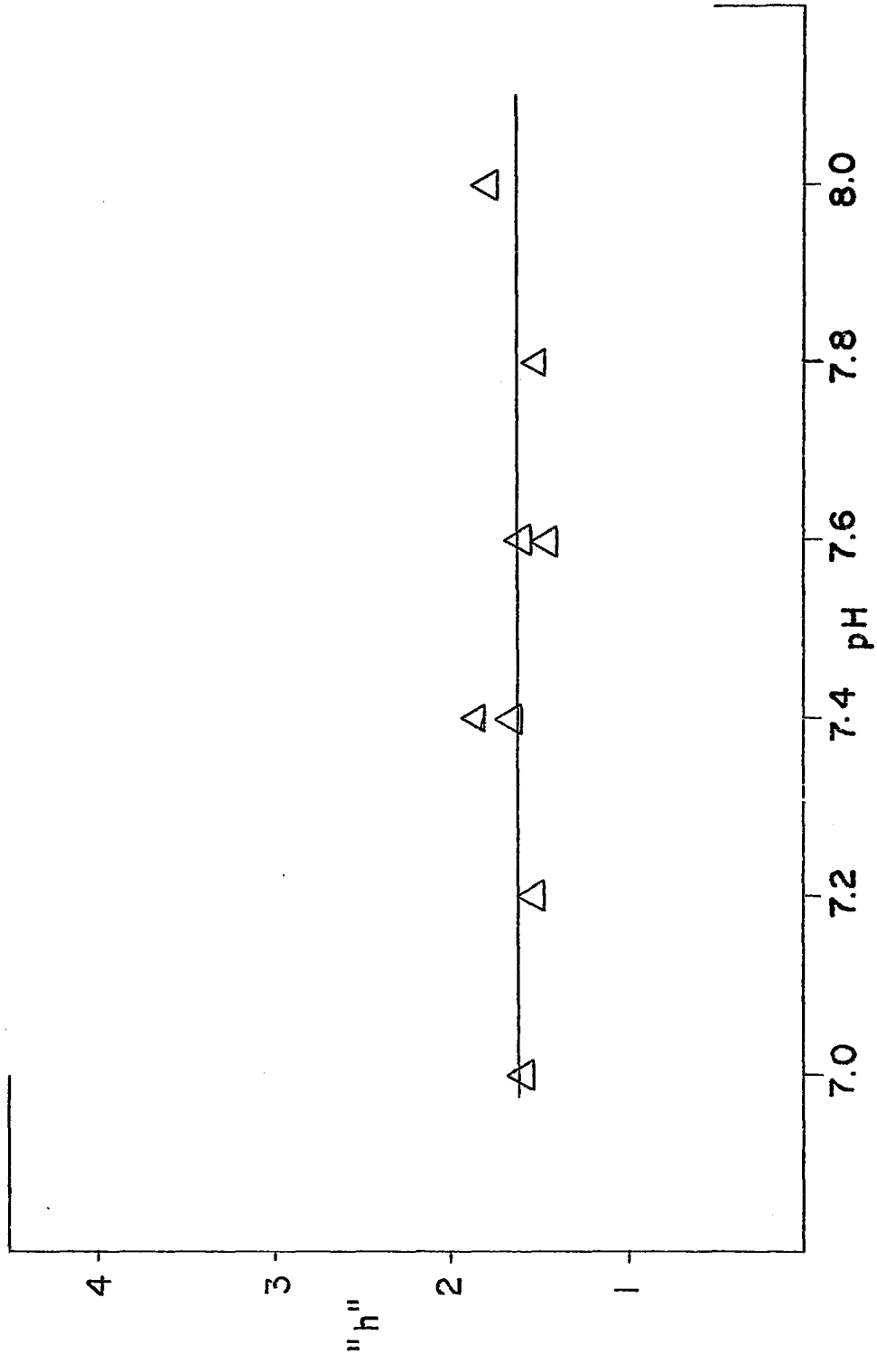


Figure 23. Effects of buffer concentration on stripped Hb A. Buffer is pH 7.4 Tris HCl. Temperature is 20° C. A and B.

A. Log P_{50} vs Log buffer concentration

B. Hill coefficient "h" vs Log buffer concentration.

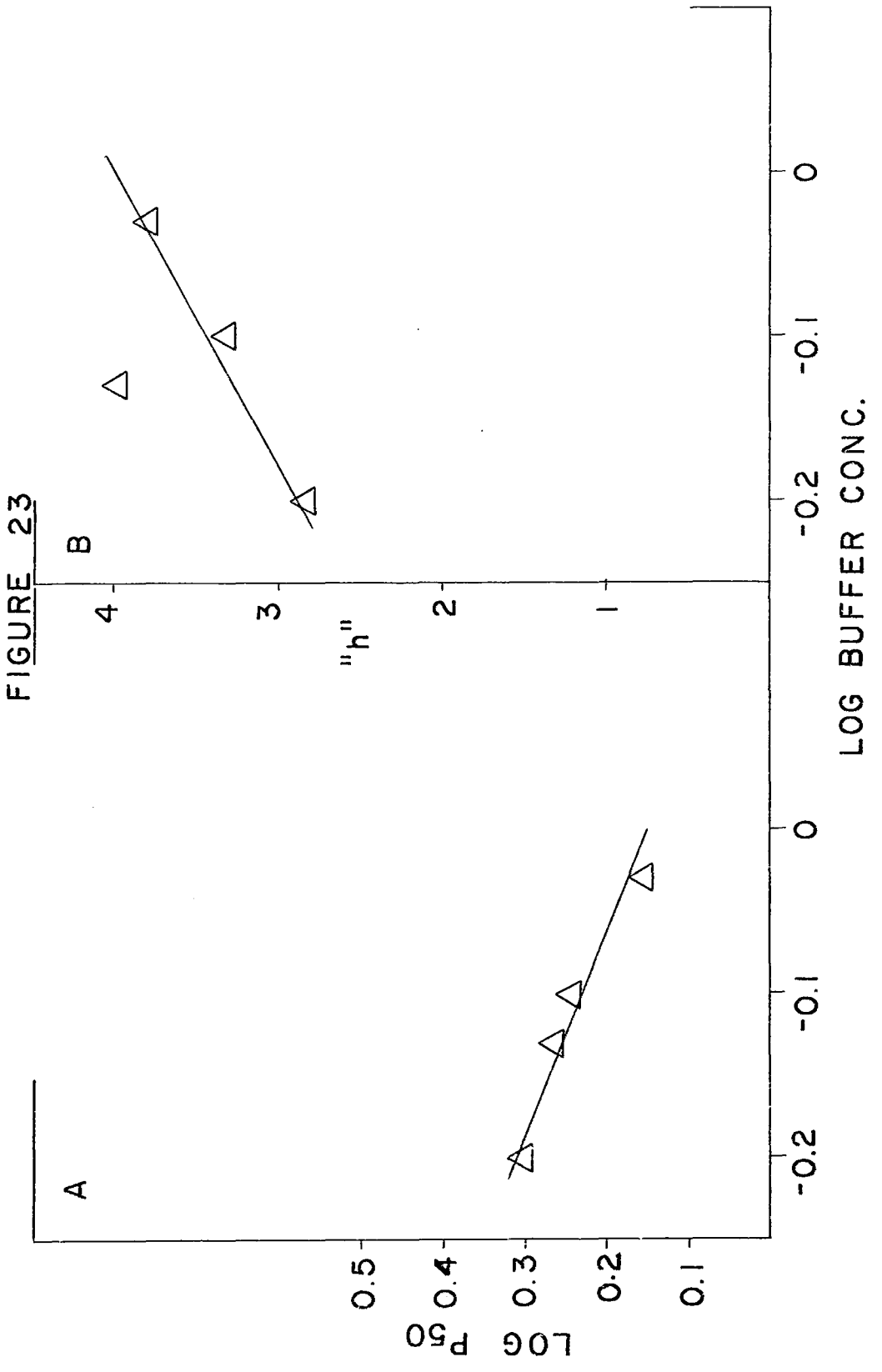


FIGURE 23

Figure 24. Effects of temperature on stripped Hb A. Buffer is 0.01 M Tris HCl pH 7.4. A and B.

A. Log P_{50} vs temperature.

B. Hill coefficient "h" vs temperature.

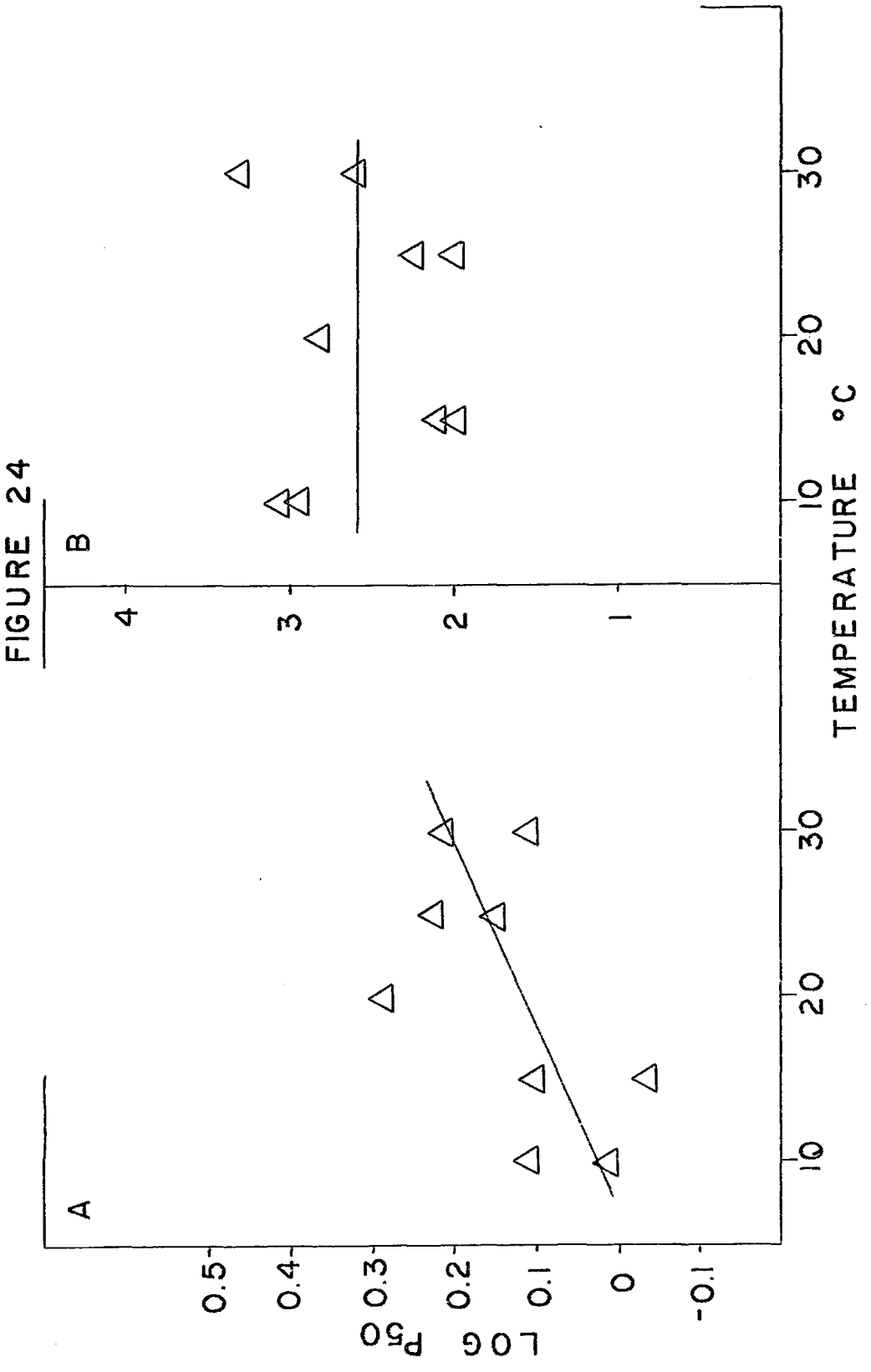


FIGURE 24

Figure 25. Comparison of the oxygen affinity of stripped murre Hb A (△——) and stripped murre Hb A with 1 mM IHP added (○——). Log P_{50} vs pH. Temperature is 20° C. Buffer is 0.01 M Tris HCl.

FIGURE 25

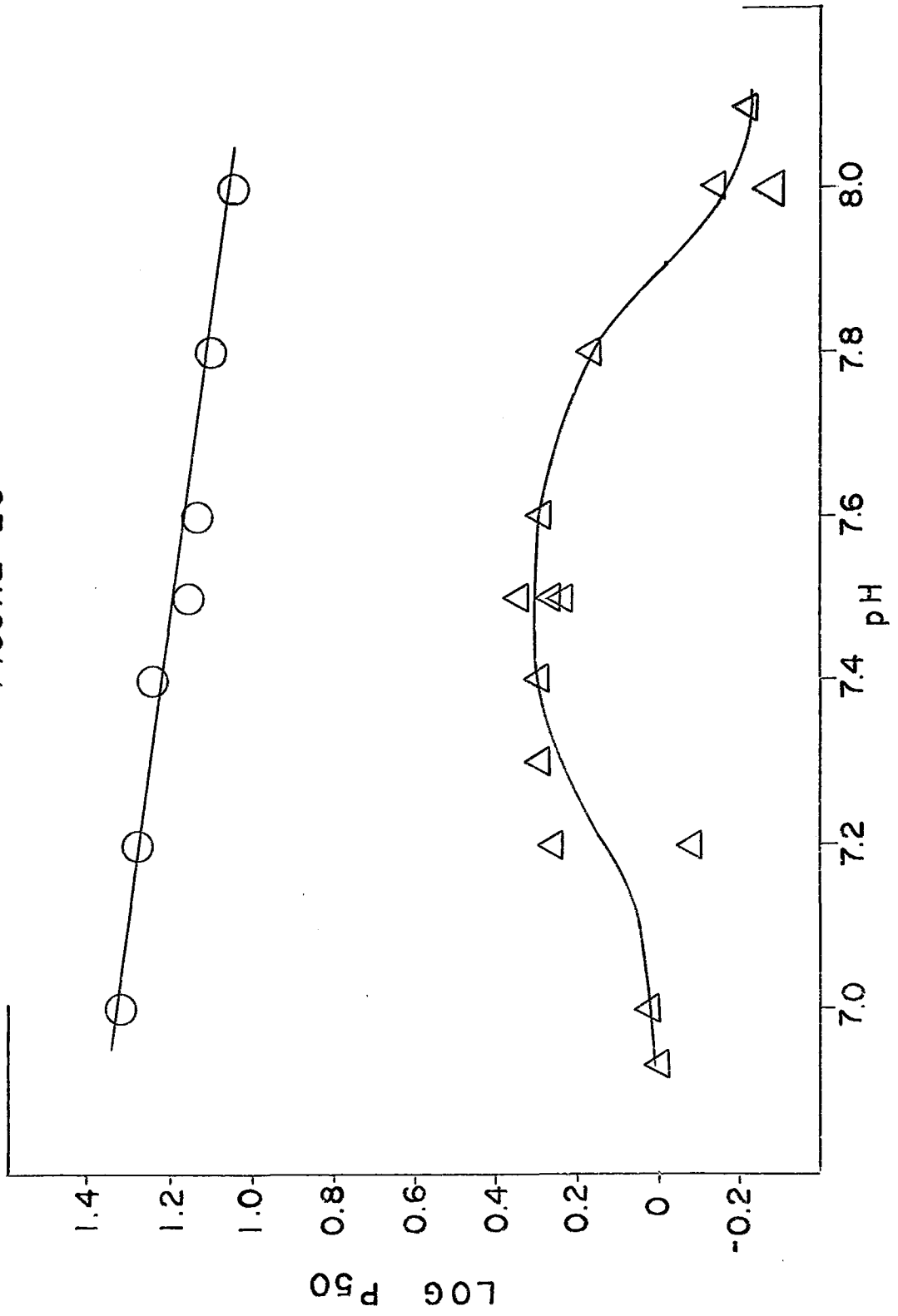
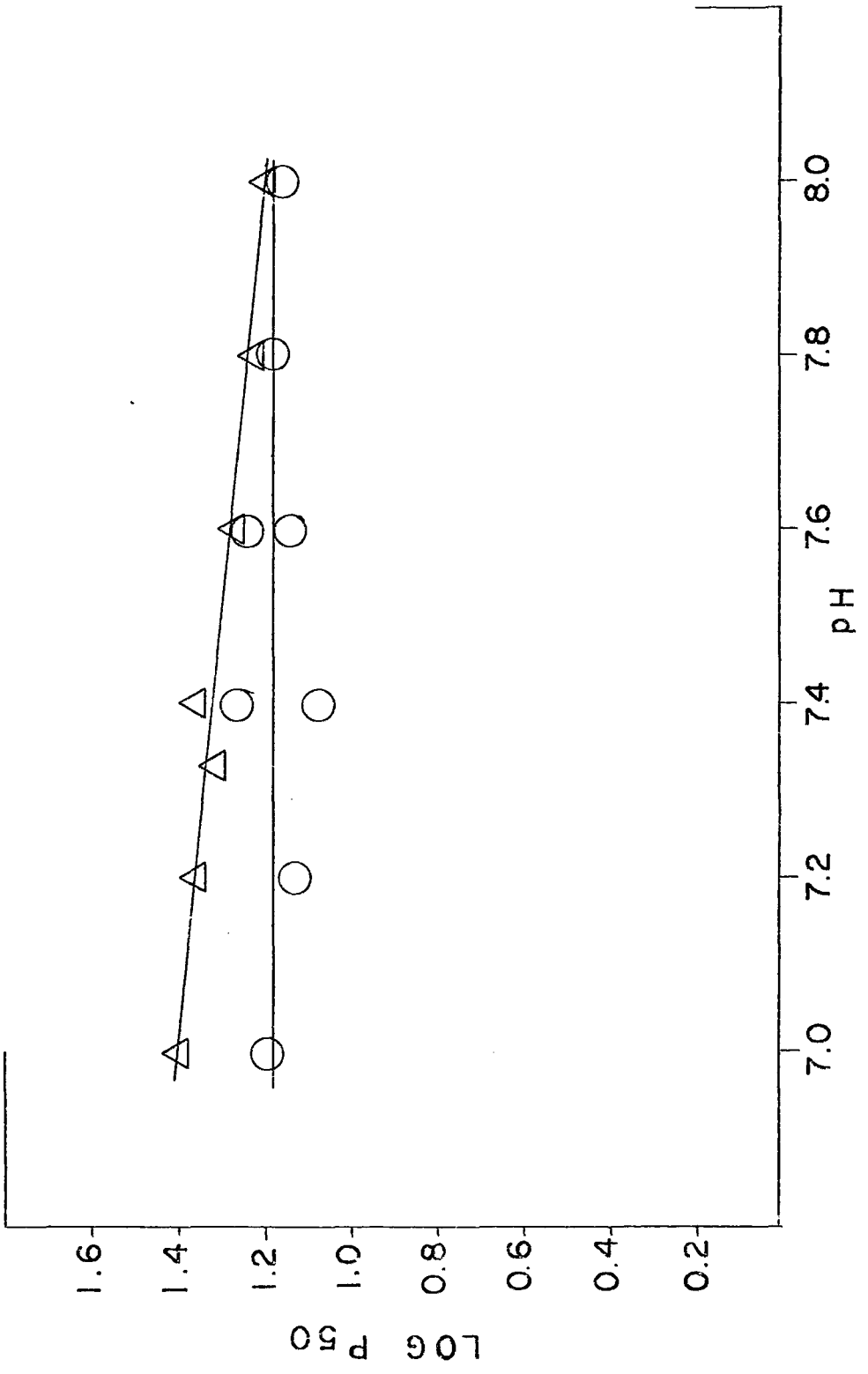


Figure 26. Comparison of the oxygen affinity of stripped murre Hb B (△——) and stripped murre Hb B with 1 mM IHP added (○——). Log P_{50} vs pH. Temperature is 20° C. Buffer is 0.01 M Tris HCl.

FIGURE 26



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