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THE pH RELATIONS OF MUSCULAR CONTRACTION

by

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THE pH RELATIONS OF MUSCULAR CONTRACTION

By Frans F. Jöbsis

The main purpose of the present study was to provide data on the pH dependence of tetanic tension production in striated muscle. The resulting data were compared to similar results in glycerin extracted muscle fibers contracting upon the addition of ATP. Certain similarities in the behavior of the two systems were noted. The effects of pH changes on the excitability were also studied and the results were found to differ fundamentally from those on tension.

Controlled intracellular pH changes were produced by first equilibrating the muscle with a given $NaHCO_3$ concentration and subsequently exposing it to varying pCO_2 levels. Intracellular pH changes were then calculated via the Henderson-Hasselbalch equation. The use of this method, which rests upon the penetration of both the bicarbonate ion and CO_2 into the cell, was necessitated by the impermeability of the muscle cell membrane to hydrogen ions. The results proved the method to be effective and also showed the presence of a fixed, intracellular base, whose buffering capacity should be added to those of the penetrating bicarbonate ions.

It was found that at 20° C the tetanic tension of the M. retractor penis of the turtle was decreased by increased acidity. This inhibition proceeds with a stoichiometric factor of 1, indicating a one to one relation between hydrogen ions and their target in the contractile system. At 0° C the same 1 to 1 relation was found in some, while a 1 to 3 ratio between hydrogen ions and the contractile system was found in other muscles.

These results were explained on the basis of complete activation of the contractile system at 20° C and partial activation at 0° C. At 20° C the muscle is considered to be completely activated and the pH effect is on the contractile properties of the proteins. At 0° C, however, activation is considered to be incomplete and the pH effect is largely an inhibition of the activation process. This interpretation was found to be consistent with the pH dependent properties of the two types of glycerinated muscle fibers in which either contractility or activation is the limiting factor in tension development.

The results on the excitabiiity showed that the inhibition of its dynamic time constant by increased acidity proceeds without the involvement of a fixed, intracellular base. This is a basic difference between the pH effects on tension and excitability. The relation between pH and the dynamic time constant of excitability proceeds on a one to one basis for the effect of the hydrogen ion on its target.

for

Sally

with all my love

voor

Vader en Moeder

met grote dankbaarheid

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CHAPTER I

INTRODUCTION

In recent years the understanding of the functional activities of cells has been greatly advanced by the practice of studying enzymes, enzyme complexes, organoids or other parts after their isolation from the cell. This method has been eminently successful in the study of the potentialities of these cellular constituents and has provided a large body of information on their properties. It may suffice here to point to our new insight in the kinetics of the cytochrome system, for example, which is derived from the study of mitochondria, prepared in reasonable purity by the method of differential centrifugation of cell homogenates. The obvious advantages of a system in which the experimental parameters are under the close control of the investigator do not need further elucidation, the accomplishments speak for themselves.

It has become the general practice to transfer these results directly to considerations of the events in the intact cell. Although in a qualitative manner this may be justified, quantitative considerations should be approached with extreme caution. In many cases, however, the transfer is made without adequate reservations about this drastic step in scientific analysis and for many systems grave doubts adhere to the resulting conclusions.

It seemed, therefore, that a useful function could be served

by an attempt at the collection of data on an intra-cellular function which had been studied extensively in a purified system. For this purpose a function has to be chosen which shows a characteristic response to analogous manipulations within the intact cell and extra-cellularly. In this way the responses of the intact system and the extracted one can then be compared and the value of an extrapolation can be judged.

The feasibility of such an analysis is obviously limited by difficulties in the controlled alteration of intracellular conditions. One system, that showed promise for this kind of analysis, consisted of the tension-developing properties of the glycerinated muscle fiber and the intact muscle in relation to pH changes. The glycerinated fiber was shown by A. Szent-Gyorgi and coworkers as well as by many other investigators to possess contractile properties in response to adenosine triphosphate which make it highly likely that this preparation should be regarded as the tension-producing mechanism of the muscle, stripped from its accompanying systems for excitation, metabolic recovery, etc.

Recently it was shown by Goodall and A. G. Szent-Gyorgyi (1953) and especially by Brown and Goodall (1956) and by Brown (1957) that the tension developed by the glycerinated fiber has a typical and specific response to relatively small pH changes, which should be recognizable in the intact cell under similar conditions.

The manipulation of the intra-cellular pH appears to be a feasible project in the light of the work by many investigators on various plant and animal cells and especially by that of Smith on cardiac muscle tissue (1926). It was, therefore, decided to attempt the study of the tension development by intact muscle as a function of intracellular pH.

The method adapted for the manipulation of the intracellular pH was first used quantitatively by Smith. It depends on the equilibration of the cell with externally applied bicarbonate concentrations and the quick penetration of CO_2 into the cell where it forms carbonic acid. In this way pH changes are effected which are governed by the Henderson-Hasselbalch equation. This devious route for varying the pH is necessary because the membrane is for all practical purposes impermeable to H^+ - ions. Both the rapid penetration of CO_2 through cellular membranes and the slower penetration of bicarbonate ions into the muscle cells of poikilotherms have been established by previous investigators (see Chapter II, p. 8 and p. 11), while Smith himself proved the feasibility of the method by his study on cardiac muscle.*

This last study showed excellent, quantitative data obtained by the application of this method. However, Smith's analysis is lacking in two aspects in their applicability to the question at hand. Smith, as mentioned, used cardiac muscle for his experimental

material and measured the isotonic shortening. Furthermore, his experimental conditions were such that the muscle was in a state of maximum treppe, which quite likely had an important effect on the response to CO_2. For purposes of comparison with glycerinated fibers, the isometric tension of skeletal muscle is a better parameter.

In view of these considerations, the present study was initiated. The approach used is identical in experimental design to that of Smith, but the analysis of the results is different. The material used was skeletal muscle, partly because of the absence of any significant amount of treppe effect and partly for the reason mentioned above. The material first chosen was the sartorius of the frog, but it quickly became evident that this tissue suffered under the treatment and soon became fatigued. Subsequent work was, therefore, performed on the M. retractor penis of the turtle, a more stable preparation. The measurement chosen was isometric, tetanic tension. This measure of the contractile system is one of a steady state and is to be preferred to the twitch tension, which is a complex function of activation and subsequent deactivation. The tetanic tension was found to be very sensitive to the correct optimal rate of stimulation. The frog sartorius, which is a quickly contracting and also a quickly fatiguing muscle is not particularly suited to the large number of contractions needed to ascertain the correct frequency of stimulation at each pH. The retractor penis,

however, will stand a far larger number of such tetani before showing fatigue, a finding which reaffirmed its position as the preparation of choice.

One of the more essential differences between the glycerinated fiber and intact muscle is the lack in the former of a selectively permeable membrane, that controls the excitation of the enclosed myofibrils. During the treatment with the glycerol-water mixture the muscle membrane breaks down and becomes freely permeable to many substances including most of the intracellular components but with the important exception of the actomyosin of the contractile elements. The resulting "skeleton of the contractile system" can be induced to contract and develop tension by the addition of ATP in a suitable ionic medium. Thus the effect of H^+ or any other agent on these fibers should be compared with the effects of these agents on tension development in the intact muscle under conditions where the excitable system is not limiting this muscle response.

Because of the importance of this last consideration in the interpretation of the H-ion effect on tension and for the purpose of comparison of a true intracellular function with a membrane phenomenon, it was decided to study the effect of H - ion on the excitability also. The necessity of verifying that the tension changes are or are not a consequence of the pH effect on excitability does not need further

emphasis. The study of the susceptibility of the membrane to H-ion concentration changes is, however, also of interest in its own right. Whereas the tension should be a function of the intracellular pH, any excitability changes may either correlate with the intra- or with the extra-cellular pH. For this study of the H ion effect on the membrane an index to the excitability will have to be found, which is reasonably stable under control conditions and which responds to the experimental parameter, i.e., pH.

In the study of muscular contraction the choice of the temperature at which experiments will be performed is an important one. The temperature curve of the tetanic tension does not present the typical picture for enzymatic reactions (see Johnson, Eyring and Polissar, 1953). In other complex, biological reactions, such as the luminescence, O_2 consumption and rate of multiplication of bacteria, hydrolysis of sugar by yeast and many others, an optimum temperature is found at which the activity in question is greater than either below or above it. At the lower temperatures the decrease is considered to be caused by a lowering of the reaction rate, while at the higher temperatures reversible denaturation of the enzymes occurs with consequent lowering of the concentration of active enzyme.

The tetanic tension shows no such behavior. It increases to a certain plateau level with the raising of the temperature above

0° C. The temperature at which this plateau is reached depends on the species studied and also shows seasonal variation. With further increases in the temperature the plateau first maintains itself but then starts to fall. Hajdu (1949, 1951) gives some curves in which a true maximum seems to be present, but upon scrutiny it was found that the result was due to irreversible denaturation, which should be eliminated from this consideration (Brown and Jöbsis, unpublished observations). Recently Brown (1957) has brought forward some interesting arguments which indicate that the decreased tension at low temperature is the result of a failure to achieve complete activation rather than a decrease of the tension produced by the activated fraction. At higher temperatures the activation increases until it becomes and remains maximal.

This situation, on the one hand, necessitates careful consideration of the choice of the experimental temperature while on the other hand it invites a study at temperatures at which either the one or the other condition is dominant. It is, therefore, valuable to determine if the effect of pH on muscle differs at low and at high temperatures. Before the correct temperature conditions can be chosen, however, the characteristic tension vs. temperature curve of the muscle under study has to be determined.

CHAPTER II

HISTORICAL AND THEORETICAL CONSIDERATIONS

A. Permeability to Acids and Bases

The problem of the action of acids on biological material is not unique for CO_2. Early in the present century, the analysis of the toxic action of many acids, mineral and organic, was the subject of extensive research. This work culminated in the view, first proposed explicitly by Harvey, (1914 a, b) and later most extensively documented by Collett (1919, 1921), that at moderately low pH (> 4) the toxicity depended on the ease of penetration of the acid into the cell, not on the external H-ion concentration. Other researchers (Crozier 1916, a, b, c, 1918; Haas, 1916), employing the pH indicator properties of certain pigments occurring naturally in some animal and plant cells confirmed this hypothesis by direct observation. In this way it was shown that the order of toxicity of acids was closely related to their ability to penetrate the cell wall and that their lethal effect was apparently exerted intracellularly. It was also suggested that the penetrating entity was the electrostatically neutral molecule rather than the anion, an hypothesis easily fitted in with existing knowledge of the low permeability of the cell membrane to anions in general.

The culmination of this work was the study on the mode of action of CO_2 by Jacobs (1920). Using anthocyan-containing stamen as

material, he proved clearly that the amazing speed of the onset of the toxic effects of carbonic acid is due to its extremely rapid penetration rate. Taking solutions of equal pH, i.e., with the same ratio of bicarbonate to CO_2, but of different total concentration of the buffer system, he showed that the intracellular pH changed with the CO_2 partial pressure. The effect was due, according to Jacobs, to a rapid rate of penetration of the CO_2, and a slow one of the bicarbonate. This anomolously fast rate of entrance into the cell was explained as being due to the penetration of CO_2 in physical solution. Once inside, the CO_2 then follows the normal scheme of hydration and subsequent ionization of the H_2CO_3, resulting in a drop of the intracellular pH.

At approximately this time another method of analysis was introduced by Smith and Clowes (1924) who used the rate of division of sea urchin eggs as the biological activity of their choice. This was followed by further work by Smith (1925, 1926) on the division of sanddollar eggs and on the isotonic shortening of turtle auricular muscle. These two physiological processes turned out to be admirably suited for a quantitative analysis of the action of various acids. Their method rested upon the fact that the buffering capacity of the intracellular moiety can be changed by manipulation of the external solution. When the eggs or the muscle tissue were allowed to stand in a solution with a certain amount of Na-salt of

the acid to be tested, some of the anions or possibly molecules of the undissociated salt would penetrate, and act as a buffer with the permeating acid molecules when the tissue was subsequently exposed to the acid. This method of analysis was successful and from it Smith concluded that the action of CO_2 on the cardiac muscle was due to its effects on the intracellular pH. More specifically, the analysis showed that the contractility of the muscle decreased with increasing pCO_2 in a fashion consistent with the concept that the intracellular pH changed as if it contained the same concentration of bicarbonate as the Ringer's bathing it plus a constant, extra amount. This extra base, termed by Smith the "fixed, intracellular" base, was found to vary from one to the next preparation, with an average value of 2.4 millimoles bicarbonate per liter. (For further discussion, see p.13 and p.17 .) Similar relations were found for other acids (valeric, butyric, propionic, acetic, lactic and hydrochloric) but with less success, because the half times for equilibrium of the fastest of these (valeric) was still approximately 20 times longer than that for carbonic acid. These long equilibration times interfered with the successful completion of the experiment.

Besides the rapid penetration of CO_2, Smith's analysis relies also on the reversible equilibration of extra- and intracellular bicarbonate. The permeability of the muscle cell membrane to this substance has in itself been open to considerable discussion in

muscle physiology. As far as mammalian muscle is concerned, the debate is not yet closed, not even to the point of the occurrence of any intracellular bicarbonate in the muscle in situ. On the one hand, Wallace and Lowry (1942) and Wallace and Hastings (1942) claim that no bicarbonate is present intracellularly in rat muscles and that none enters after excision and exposure to various bicarbonate concentrations from 0 to 87 mM. Conway and Fearon (1944) on the other hand, show that approximately 1 mM is intracellular in rabbit muscle, while in 1941 Boyle and Conway showed that the intracellular concentration varied with the extracellular one. Creese (1949, 1951) came to the conclusion that the improvement in the contractility of the excised rat diaphragm upon addition of bicarbonate to the Ringer's can not be explained on the basis of external pH changes. His experiments strongly suggest a penetration of the bicarbonate into the cell. It must be concluded that the dispute has as yet not been settled.

For cold-blooded animals the situation is quite different. Here the work of many investigators has shown that a considerable amount of bicarbonate is present in their muscles and that the cell membrane is permeable to it. Of these studies the direct measurements by Brookens (1933), Fenn and Cobb (1934) and Fenn and Maurer (1935) are the most conclusive. The first author showed that considerable bicarbonate is present in muscle in situ and especially that this level becomes directly dependent and equal to the concentration in

the Ringer's solution upon excision. Fenn and his coworkers extended this work and verified the conclusions in general, with the exception that they noticed a small but consistent surplus of HCO_3^- in the Ringer's so that the ratio of the intra- to the extracellular level is as 1 to 1.2 (see also Fenn, 1936). This proof of its entrance into the cell and its participation in the internal buffering capacity is corroborated by numerous reports on the beneficial effects of bicarbonate on the function of excised muscle tissue (Berg, 1911, Rona and Neukirch, 1912). The results of Smith's study (1926) concerning the penetration of bicarbonate are, therefore, in good agreement with the known behavior of the muscles of cold blooded animals.

However, the amount of HCO_3^- which at equilibrium has penetrated from the Ringer's can not be found from his method. Complete equilibrium, a constant fraction or even a multiple of the external solution are all possibilities, though this last one must be considered unlikely in view of Fenn's work. Accordingly, the fixed intracellular buffer fraction mentioned above can not be considered as being equal in buffering capacity to 2.4 mM bicarbonate, but rather to the same amount of bicarbonate as would enter the cell upon an increase of the external level by 2.4 mM. The actual, chemical make-up of this buffer fraction is completely undisclosed. It is more than likely, however, that it enters in some fashion in the internal buffering capacity of the cell.

B. The Buffering Capacity of the Muscle Cell

A good number of investigations have touched upon the buffering power and other pH relations of the muscle cell, but the results unanimously indicate a much larger intrinsic buffering capacity than the 2.4 mM found by Smith. Many of these studies have involved the determination of the CO_2 binding capacity of the muscle in analogy to the work on whole blood (Fenn, 1928; Stella, 1929; Irving, Foster and Ferguson, 1932; Cowan, 1933; Root, 1933; Fenn and Maurer, 1935; Wallace and Hastings, 1942; Wallace and Lowry, 1942; see also Meyerhof, Mohle and Schultz, 1932). Similar studies employing the uptake of NH_3 have also provided data on the buffering capacity of the cell (Netter, 1934; Fenn, Noonan, Mullins and Haege, 1941), while the most direct information was derived by Furusawa and Kerridge (1927) and Mackler, Olmsted and Simpson (1930) who titrated muscle brei and measured the pH changes. This last work, however, is open to the objection that the method does not leave the cells intact and thereby introduces errors. All these methods indicate buffering capacities considerably greater than the one found by Smith.

Because of the widespread application of the first method, it may be valuable to consider that technique in some detail. When a solution of unknown buffering characteristics at a pH of about 7 is exposed to a given pCO_2, the increased concentration of $H^{\oplus}$ - ions will be neutralized by the buffers present, leaving an excess of

HCO_3^- ions, associated with the cations set free from the basic member of the buffer system. This process decreases the H_2CO_3 concentration so that new CO_2 can dissolve until equilibrium is reached. If an excess of strong acid is now added to this solution, the increased H^+ - ion concentration will drive the dissociation equilibrium of the H_2CO_3 back to the undissociated form and finally to physically dissolved CO_2 with the consequence that CO_2 will be evolved. Manometric measurement of this CO_2 gives us the amount of HCO_3^- present at the time the acid was added. (Strictly speaking a number of assumptions have been made as to the negligibility of the concentration of CO_3^-, the changes in solubility of the CO_2 in physical solution at sharply different pH values, etc. which will here be taken for granted.) From the HCO_3^- concentration and the pCO_2 the pH can now be calculated by the Henderson-Hasselbalch equation, provided the pK_1' is known for the particular conditions of the experiment.

In the case of muscle, a carefully excised and weighed muscle is introduced in a manometric vessel, which contains acid in a side arm, and is equilibrated with a gas mixture of known pCO_2. The system is then closed and the acid tipped on the muscle. The evolved gas is measured and the intracellular pH is calculated, making certain provisions for the Ringer's contained in the intercellular spaces and for the solids present in the tissue.

From such measurements at varying CO_2 partial pressures the

buffering power of the cellular fluid can be calculated. For this purpose we can use van Slyke's (1922) definition of buffering power as

$$\beta = \frac{dB}{dpH} = \frac{\text{g. equiv. base or acid added to 100 ml of fluid}}{\text{resultant change of pH}}$$

where, by definition, the acid or base used to bring about the pH change is a strong one, i.e., completely ionized in dilute solutions near neutrality. From this definition it follows that when β is large, substantial amounts of acid or base have to be added to produce the pH shift, i.e., the solution is well buffered. When a relatively weak acid is used, such as carbonic acid in our case, the actual H^+ - ion concentration produced by the dissociation should be used for the calculation, not the total amount of acid. This amount can be calculated from the difference in the volume of CO_2 taken up at two different partial pressures and the pH calculated as outlined in the previous paragraph. This difference in the CO_2 evolved is then equal to the amount of H^+ - ions neutralized in the muscle. The data of Stella (1929) for frog muscle are probably most applicable to Smith's work and the study proposed here because of the similarity in material and in the time of exposure, which was shown to be extremely important by Root (1933). From Stella's work it can be calculated that for the fiber water of the tissue β is equal to 0.027 for the range of pH from 6.92 to 6.61, as produced by an increase of the pCO_2 from 25 to 100 mm Hg.

This compares reasonably well with the value of $\beta = .025$ for cat striated muscle over the same pH range measured directly by titration of the brei with lactic acid. The buffering capacity of the 2.4 mM HCO_3^- of "fixed intracellular buffer" produces a value of only 0.0004 over the same pH range. This discrepancy is far beyond any that can be ascribed to experimental errors in either technique. It may be worth while to look for possible factors which could cause this difference.

The difference in biological material must definitely be considered as a possible source of a discrepancy. Furusawa and Kerridge's study (1927) established, for instance, large differences between the striated, cardiac and smooth musculature of one species: the cat. At pH 7.0 the three β values are respectively 0.025, 0.013 and 0.019. It is, therefore, quite probable that large differences exist between cat, frog and turtle and between the gastrocnemius and cardiac muscle.

Furthermore, Stella's data on the frog gastrocnemius were made by a procedure which makes it unlikely that the 10 to 11 millimoles of bicarbonate per liter of fiber water, which is present in fresh muscle, had diffused away. This additional bicarbonate could have produced an extra buffering capacity up to a maximum of a β value of about 0.002. Subtracting this amount, we would arrive at $\beta = .025$ for this muscle.

The question of the formation of carbamino and other compounds

should also be considered. The CO_2 dissociation curve provides data on all the CO_2 which enters the muscle. The chemical state in which the CO_2 is present in the muscle is, however, not necessarily that of bicarbonate only. It is well known that a considerable proportion of the CO_2 carried in the venous blood is bound to hemoglobin in carbamino compounds, and that this fraction does not participate in the acid-base balance. One study has dealt directly with this aspect in muscle. Conway and Fearon (1944) found that in rabbit and cat leg muscle 66% of the CO_2 set free by acid treatment was not precipitated by barium hydroxide and could, therefore, not have been present in the form of CO_2 or HCO_3^-. They suggested that about 30 to 40% of this fraction was in carbamino form, while the rest must have been present as unknown compounds. After allowance for the serum bicarbonate in the intercellular spaces, they concluded that approximately 0.9 mM of a total of 10.6 mM, found in whole muscle by the CO_2-evolution method is present as bicarbonate in the intracellular milieu. It may be useful to emphasize once more that the 2.4 mM of "fixed intracellular buffer" found by Smith would represent the total buffering capacity of the contractile system of the cell as revealed by the functional analysis performed in that study. The 0.9 mM found by Conway and Fearon is only the contribution of the intracellular bicarbonate to the total buffering capacity.

This factor of approximately 10 found by Conway and Fearon,

when applied to the values of whole cat muscle, would produce values of 0.0025, 0.0013 and 0.0019 for mammalian striated, cardiac and smooth muscle respectively. For Stella's data on the frog gastrocnemius this would lower the β from 0.025 to 0.0025.

It is clear that a considerable discrepancy still exists between these values and Smith's β value of 0.0004. An added interest of the proposed study will, therefore, be the provision of data for comparison with that value.

C. The Intracellular pH

The question of the average intracellular pH of muscle tissue is as yet unsolved and can, therefore, not be used for the fixing of the pH scale in the last part of the proposed study. Compilations of the literature have recently been made by Wiercinsky (1955) and by Caldwell (1956), a fact which greatly facilitates the reviewing of the field. From these two articles it is at once evident that a great discrepancy exists between the results of the two most commonly used methods. CO_2 dissociation curves and indicator studies. The first of these has been described extensively above, the intracellular pH being calculated from data on the CO_2 pressure and the CO_2 released by acid via the Henderson-Hasselbalch equation, after the assumption of a pK_1' value.[1] The values for

1. The common practice of assuming equal pK_1' values for intra- and extracellular solutions at equal ionic strength will be followed here. Meyerhof, Möhle and Schultz assumed a different pK_1 value under intracellular conditions on the assumption that the intracellular pH should equa the extracellular one. This practice is unsupported by sufficient evidence.

the intracellular pH found from these calculations vary from 6.9 to 7.2 for various mammalian, frog and crab muscles.

Two criticisms should be leveled at these studies. The first one was originally pointed out by Conway and Fearon (1944), who showed that the majority of the CO_2 set free by acid was not present in the muscle as free HCO_3^- but in bound form and can therefore not be considered to participate in the buffering reactions. They determined the amount of this so-called barium-soluble CO_2 and calculated an internal pH of 6.0 for rabbit leg muscles. Notwithstanding this clear demonstration, very little heed has been paid to this grave criticism by subsequent reviewers. A. V. Hill (1955) does pay attention to it, however, and tries to bring their data in line with the vested values by an unconvincing calculation from other data of these authors. (This calculation is quoted in a misleading manner by Caldwell in his compilation.) However, no effort to either prove or disprove Conway and Fearon's contention on the basis of new experimental data has come to this author's attention.

The other criticism stems from the work of Root (1933) who found that the amount of CO_2 released from muscle by acid treatment increased with increases in the duration of the preceding CO_2 equilibration period. His work shows that the maximum amount of CO_2 is released from frog muscle, only after exposure to CO_2 for 5 hours, while at 3 hours and 1 hour the amounts are only about 80% and 50% respectively. Root interpreted this finding

on the basis of a slow phosphocreatine breakdown. It means, however, also that the buffering capcity of the cell increases with the period of exposure to CO_2, or in other words, that the pH calculated for the intracellular milieu increases with the time of exposure. This fact has not always been taken into account in discussions of the intracellular pH as derived from CO_2 dissociation curves.

Another reason for caution towards this method stems from the analogous use of NH_3 as a penetrating base. This method, which is completely symmetrical to the CO_2 method, should arrive at an identical, intracellular pH. Netter (1934) finds, however, a pH of 6.5 while Fenn, Haege, Sheridan and Flick (1944) come to the conclusion that it is 6.6. It should be pointed out that the same senior author decided that the internal pH was equal to 6.9 by the use of the CO_2 method (Fenn and Maurer, 1935). It seems apparent that several unidentified as well as some recognized pitfalls adhere to the method of pH determination by equilibration with penetrating acids or bases.

The second method, that has been applied extensively for the determination of the intracellular pH, involves the use of indicator dyes either by microinjection or by vital staining. The usual Clark indicators are commonly used, because they were shown to be negligibly affected by proteins. In the microinjection technique a particle of dye is introduced into the cell with a micropipette

and the color matched to that of a similar particle in a drop of buffer solution of known pH. Results with this method have produced a range of values from 6.5 to 6.9. It may be pointed out, however, that the data probably relate mostly to the more dilute and more structureless parts of the cell, while it seems likely that the particles were excluded from the dense myofibrils themselves.

The vital staining technique has produced more acid values, most of them below pH 6.0. It seems that this method is free from one possible error of the indicator particle method in that there is no reason to believe that the dye should not penetrate to all parts of the cell and, thereby, indicate a truly average pH value.

The indicator methods have been attacked principally on two grounds. On the one hand, the discrepancy of the results with those produced by the CO_2 method has been ascribed to so-called protein errors, i.e., the presence of proteins affecting the indicator in such a way as to produce an erroneous pH value. This objection seems to be quite unfounded because of the absence of any sizable protein error in the indicators used, as experimentally verified first by Clark and afterward by virtually every investigator in the field. The other objection, usually leveled at the vital staining technique, denies the penetration of these indicators through the cell-membrane into the cell. This opposition seems to be rather shortsighted, because we are then left with the necessity of explaining a whole pH unit's difference between the

same Ringer's solution outside the muscle and in the spaces between the muscle fibers, where the dye must then be located.

Finally, the recent method of introduction of a micro-pH-electrode into giant muscle cells as developed by Caldwell (1954, 1955) may be mentioned. Using a glass electrode plus reference electrode assembly of total diameter of 100 μ he explored the pH relations of crab muscle fibers of 600 μ diameter and decided that the internal pH was 6.9 to 7.1. This method is, however, open to the objection that the insertion of this still relatively large, foreign body into the muscle fiber may cause considerable damage. Though many of the fibers did not seem to be visibly damaged, the possibility of severe injury is extremely likely. From Caldwell's data it can be calculated that the average resting potential of the fibers penetrated by his pH electrode was 29.5 mV. Fatt and Katz (1953) found, however, a resting potential of 70 mV for the same preparation, using Ling-Gerard electrodes of standard micro-dimensions (1.0 μ diameter). Consideration of this discrepancy shows Caldwell's data to be unacceptable.

From all these considerations it must be concluded that no definitive value can be assigned to the pH of the muscle cell.

D. The Reactions of CO_2 with Water

When CO_2 dissolves in water the following reactions are considered to take place:

$$CO_2 + H_2O \rightleftharpoons H_2CO_3 \quad (1)$$

$$H_2CO_3 \rightleftharpoons HCO_3^- + H^+ \quad (2)$$

$$CO_2 + OH^- \rightleftharpoons HCO_3^- \quad (3)$$

$$HCO_3^- \rightleftharpoons CO_3^{=} + H^+ \quad (4)$$

It would be well to consider these four reactions, to their extent as well as their speed of approach to equilibrium.

The last of these four equilibria has a dissociation constant $K = 1.8 \times 10^{-11}$, which corresponds to a pK of 10.40. From this it follows that at any pH below 8, the hydrogen ion concentration becomes so large and the equilibrium driven to the left to such an extent that the degree of dissociation of HCO_3^- becomes less than 1%, as first clearly pointed out by E. J. Warburg in 1922. The occurrence of reaction (3) was established by Faurholt (1924) who found it to be of appreciable import in fairly alkaline media, but of no consequence near neutrality. In this study, where the pH never exceeds 7.4, the contributions of reactions (3) and (4) can, therefore, be neglected. Equations (1) and (2), however, play an essential role in determining the pH of a solution containing CO_2.

The amount of carbon dioxide that dissolves in water depends, according to Henry's law, on the CO_2 tension in the gas phase above it. This law states that "the mass of gas dissolved by a given volume

of solvent, at constant temperature, is proportional to the pressure of the gas with which it is in equilibrium" (Glasstone, 1946). Although strictly true only for ideal gases, the deviations produced by the non-ideality of CO_2 do not become significant until pressures of many atmospheres are employed at elevated temperatures. Another possible source of deviation would seem to lie in the fact that part of the CO_2 becomes hydrated to form H_2CO_3 in accord with reaction (1). However, only the CO_2 in physical solution is involved in considerations of the applicability of the law. Because during the course of the present experiments the gas mixtures were continually bubbled through the solution and, therefore, any fraction of the CO_2 converted by reaction (1) was replenished immediately, it is justified to apply Henry's law. This was experimentally proven by the work of Harned and Davis (1943) for water and for NaCl solutions of .1 to 3.0 M over the temperature, pressure and pH ranges employed in this study. From here on it will, therefore, be warranted to proceed from the basis of a direct and constant proportionality between the different partial pressures of CO_2 above a given solution and the CO_2 dissolved in it.

The rate of solution of CO_2 into water is a relatively slow process unless a large surface area for gas exchange is provided. It was found empirically that when the gas was bubbled through

as a stream of small bubbles by forcing it through a porous, plastic "aquarium-stone" at the bottom of the vessel, the pH of the Ringer's came to a stable value (.01 pH units as measured with the Beckman model G S pH meter) in 10 to 15 minutes. The period of aeration was, therefore, never allowed to be shorter than half an hour, while usually it was considerably longer. To this period should yet be added the 15 minutes needed for the equilibration of the muscle with each new solution, during which period a gentle stream of bubbles was passed through the Ringer's in the muscle chamber. These periods of equilibration insure that the deviation from equilibrium at the time of the stimulation of the muscle was far smaller than any whose effect might have been discernible in the muscle's response.

Reaction (1) describes the hydration of CO_2 resulting in the formation of carbonic acid. The position of this equilibrium is far to the left with only about 0.1% of the CO_2 being transformed, as noted above. The speed to the attainment of this equilibrium was calculated by Faurholt in 1924. He found that at 18° C 90%, 99% and 99.9% approach to equilibrium was completed in respectively 79, 158 and 237 seconds at pH 7.4. These times are materially decreased by various catalysts (Roughton and Booth, 1938) three of which are present in the cell and, therefore, interest us here especially, in view of the possibility that the CO_2 has its effect

by penetrating the cell and there participating in the acid-base equilibrium. These catalysts are (1) phosphate ions (2) substances with both a straight chain and a cyclic nitrogenous base, such as histidine, and (3) the enzyme carbonic anhydrase. The presence of free phosphate ions as well as the occurrence of substances included in (2) above is considered to be a well established fact. The presence of carbonic anhydrase in skeletal muscle tissue of the red variety was established by van Goor (1940) for the rat. The white muscles, however, seem to be devoid of this enzyme. As far as the retractor penis, a red muscle, or any other muscle tissue of the turtle is concerned, no data are available.

The occurrence of the first two catalysts and the possibility of the presence of the third one both tend to lower the time needed for the reaching of equilibrium conditions in the 10 minutes allowed during the experiment. This correlated well with the empirical finding that the changes in muscle-tension production have come to a stable value in approximately 6.8 minutes (see "Methods").

The exact extent of equilibrium (1) is of fundamental importance to the problem at hand, as the concentration of carbonic acid directly influences the concentration of H - ions by the next equation. This direct connection between the CO_2 partial pressure and the pH of a given solution, together with the experimental difficulty in determining the actual H_2CO_3 concentration in the presence of a large excess of CO_2 in physical solution, gave rise, at an

early date, to the practice of telescoping equations (1) and (2) to produce

$$CO_2 + H_2O \rightleftharpoons H^+ + HCO_3^- \qquad (5)$$

In writing the equilibrium constant for this reaction the H_2O concentration changes can be neglected and the expression becomes

$$K = \frac{{}^{a}H^+ \times {}^{a}HCO_3^-}{{}^{a}CO_2} \qquad (6)$$

where the "a"'s denote the activities of the three components. This constant K is often erroneously referred to as the first dissociation constant of carbonic acid and is written as K_1. In reality, however, it differs from the "true" K_1 by a factor equal to the extent of the hydration of the CO_2, i.e., approximately 10^{-3}.

In connection with work on the acid-base relations of the blood, L. J. Henderson (1909) introduced another simplification. For practical purposes it is advantageous to substitute actual concentrations for the activities of the three components which, however, has the consequence of making the value of the dissociation constant dependent on the ionic strength of the solution. Consequently this new constant,

$$K_1' = \frac{[H^-]\ [HCO_3^-]}{[CO_2]} \qquad (7)$$

is equal to K_1 only at infinite dilutions of all components, and K_1' has to be determined for the ionic strength of the system at hand.

In 1916 Hasselbalch introduced the practice of expressing the Henderson equation in logarithmic form and calling the negative logarithm of K_1' the pK_1' of carbonic acid in analogy to the pH. The resulting expression

$$pH = pK_1' + \log \frac{[HCO_3^-]}{[CO_2]} \qquad (8)$$

has found widespread application and is generally known as the Henderson-Hasselbalch equation.

Since the introduction of these modifications and simplifications, several determinations of the actual H_2CO_3 have been made enabling us to calculate the "true" first dissociation constant of carbonic acid, which will be designated K_{1a}', and is defined by

$$K_{1a}' = \frac{[H^+][HCO_3^-]}{[H_2CO_3]} \qquad (9)$$

The several attempts at the determination of this constant in the presence of CO_2 and HCO_3^- have produced a rather wide variation of values. None of these seem to have much to recommend themselves over the others except the determinations of Roughton. In 1936 Roughton first attempted to solve this problem by the application of heat measurements in the first 1/100 second after the mixing of a solution of $NaHCO_3$ and CO_2 with HCl, using his elegant, rapid-flow technique. Slight modifications and improvements

of this method resulted in 1941 in data indicating values of the pK_{1a} (i.e., pK'_{1a} at infinite dilution) of 3.60, 3.53, 3.49 and 3.47 at 0, 15, 25, and 38° C respectively. In spite of the availability of these data, the classic pK'_1 will be used in the subsequent discussions because of standard practice and because more extensive determinations have been made over the relevant ranges of temperature and ionic strength.

For the determination of the pK'_1 the pH, HCO_3^- concentration and the CO_2 concentration must be known for the particular conditions at hand. The pH can be measured with a glass electrode or preferably with the standard hydrogen electrode. For the HCO_3^- concentration the total salt should be used because of Henderson's simplification. The amount of CO_2 in the solution must be determined, usually by volumetric methods. From these three data the pK'_1 can then be calculated for the particular conditions of ionic strength and temperature of the solution. It may be well to mention that the pK'_1 does <u>not</u> depend, under the conditions of these experiments, on the CO_2 partial pressure. This is due to the fact that 99.9% is in physical solution and, therefore, does not contribute appreciably to the ionic strength. It should also be realized that a possible effect of the ionic strength and the temperature on the degree of hydration of the CO_2 should not be considered separately, as any possible change is already reflected in the pK'_1. The small amount of additional HCO_3^- formed by the dissociation of the carbonic acid

can be neglected when the H^+ - ion concentration is .01 of the bicarbonate concentration. In other words, at pH 7.0 the $NaHCO_3$ level should not be below .01 mM, while at pH 6 the level should be .1 mM and at pH 5 at least 1.0 mM, for the contribution from the H_2CO_3 to be negligible.

Because of the importance of the HCO_3^- and CO_2 relations to the function of the blood, a great number of determinations of the CO_2 solubility and the pK_1' have been made, under varying conditions of temperature, pCO_2 and μ. Of these the work by Harned and coworkers (Harned and Davis 1943, Harned and Bonner 1945) must be preferred. These authors determined both CO_2 solubility and pK_1' for a temperature range from 0 to 50° C at 5 degree intervals for NaCl solutions of 0.0, 0.1, 0.2, 0.5, 0.7, 1.0, 2.0 and 3.0 M. Their results, therefore, eliminate the necessity of extrapolation over wide ranges, while their use of the standard hydrogen electrode as a measuring device also makes these data particularly well suited to our problem. From their data the pK_1' values and the CO_2 concentrations used in this study, have been found by graphical interpolation at .125 M NaCl. In table I these values are listed together with several other determinations.

These other data are almost invariably reported as pK_1, i.e., the extrapolated value for pure water, and therefore require a correction for the ionic strength. For this factor three values have found considerable application. E. J. Warburg (1922) suggested

Table I

VALUES FOR pK_1' AND THE SOLUBILITY OF CO_2 AT DIFFERENT TEMPERATURES

	pK_1'										
Reference	0°	5°	10	15	20	25	30	35	38	40°C	
Harned & Bonner '45	6.38	6.27	6.21	6.17	6.13	6.10	6.07	6.05	6.04	6.03	1*
Kauko & Ely '39	6.43					6.21					2
MacInnes & Belcher '35						6.10			6.04		2
Shedlovsky & MacInnes '35	6.38			6.20		6.12			6.04		2
Stadie & Hawes '28					6.18				6.09		3
Kendall '16	6.46					6.21					2
Walker & Cormack '00					6.26						2
	mM CO_2 at p = 1 atmosphere										
Harned & Davis '43	74	62	52	44	38	33	29	26	24	23	1

1.* Interpolated for 0.125 M NaCl. 2. From pK_1, corrected to 0.125 M NaCl. 3. At μ = .170.

a value of $-.46\sqrt[3]{C}$ (where C equals molar concentration of the cations) while Saunders (1926) found $-.53\sqrt[3]{C}$. Taking a middle value of $-0.50\sqrt[3]{C}$ this correction decreases the pK_1' value for pure water by .25 units for a Ringer's solution with $\mu = 0.125$. MacInnes and Belcher, however, arrived at a figure of $-.08\mu$ as a correction factor, which produces a decrease of only .01 units. From the data of Harned and Bonner the difference in pK_1' between pure water and .125 M NaCl solution are -0.20 at 0° C, -0.25 at 20° C and -0.27 at 40° C. The corrections of Warburg and Saunders are, therefore, in good agreement with these results, and for table I, a correction of -0.25 at 25° C and one of -0.27 at 38° C has been applied to the pK_1 values determined for pure water.[2]

Once the values of the pK_1' have been determined, the pH of any bicarbonate solution can then be calculated by equation (8) if the bicarbonate and CO_2 concentrations are known. As before, the first quantity is simply that of the amount of the bicarbonate salt dissolved in the solution. The amount of CO_2 that will dissolve is, however, a complex function of the CO_2 partial pressure, the ionic strength and the temperature. Harned and Davis have determined the effect of each of these three parameters and found that

2. Because of the widespread use of Umbreit, Burris and Stauffer's excellent laboratory manual "Manometric Techniques and Tissue Metabolism" (1949), it may be valuable to mention that it advocates the incorrect factor of -0.08μ.

up to 1 atmosphere Henry's law is strictly applicable in both pure water and in any given salt solutions. Contrary to the common tacit assumption that the ionic strength does not influence the CO_2 solubility, they found that there was an effect, especially at the lower temperatures. The values for 0.125 , listed in the lower half of table I, were found by interpolation from their results. It should be pointed out that these data are the direct experimental results obtained from gas analysis; they are not corrected for the effect of the water vapor pressure or its changes with changing temperatures or ionic strength. It is, therefore, unnecessary to introduce a vapor pressure correction when using these data.

The amount of CO_2 that dissolves in water from a certain CO_2 and O_2 mixture is also dependent on the atmospheric pressure. The data in table I are all calculated for the standard pressure of 760 mm Hg. The concentration for any other ambient pressure can easily be calculated because of the strict proportionality between dissolved CO_2 and pressure. In this study the quantitative experiments for which this factor might have been of importance were all performed with relatively low CO_2 tensions (maximally 30%). At an extreme barometer variation of plus or minus 25 mm Hg the difference would be less than 1% CO_2 for the 30% case and for gas mixtures with less CO_2 proportionally smaller. Although this correction could easily have been applied, it was decided to omit doing so because it was considered to be more advantageous to

pool the data for each gas mixture and use the average for calculations of the degree of inhibition (see figure 3). This practice must have contributed slightly to the experimental error.

CHAPTER III

MATERIALS AND METHODS

A. Materials

The sartorius muscle of male frogs (Rana pipiens) were used for some experiments but the bulk of the observations were made on the retractor penis muscle of the painted turtle, Chrysemys picta. This muscle is extremely durable and gives good tensions even three to four days after excision, provided it has not been subjected to extreme conditions of pH, or high temperatures. It is a long, cylindrical muscle of the striated type. The size varies considerably, even between specimens of approximately equal external measurements. The cross-sectional areas are in the order of a few mm^2, which compares favorably with the frog sartorius and assures good rates of oxygen and CO_2 exchange. The extracellular space communicates freely with the external solution as evidenced by the short time (5-10 min) needed for the stabilization of the response to agents introduced into the external fluid.

The origin of the muscle is on one of the vertebrae of the lumbar region while it inserts on the tough connective tissue of the penis. It passes between several muscles but has its own connective tissue sheath and is easily dissected without injury. It can be excised with fragments of the vertebra and the penis, leaving its attachments intact. Mechanical connections can be made through these inert tissues. (For a more detailed description see Hoffmann,

1913, and Bishop and Kendall, 1929).

The standard Ringer's solution used had the following composition:

NaCl	113.0	mM
KCl	5.0	"
$CaCl_2$	2.0	"
$NaHCO_3$	5.0	"

ionic strength = $\mu = 1/2 \sum c_i z^2_i$

$= 0.126$

where c_i = concentration of each ion
z_i = its valence

and was equilibrated with various CO_2 partial pressures in O_2. When the bicarbonate concentration was varied, or when during some experiments a phosphate buffer was used, then the ionic strength, μ, was kept constant by adjustment of the NaCl concentration. In calculations of the pH from the partial pressure of CO_2 and the bicarbonate concentration pK'_1 values of 6.13 for 20° C and of 6.38 for 0° C were used. The justification of this is given in Chapter II, section D.

The muscle was fastened vertically in a Ringer's bath in a thin plastic cylinder. The connective tissue of the penis was tied to a sturdy glass hook, while the other end of the muscle was connected to an isometric torsion lever through a light, metal chain. The tension was recorded using an optical lever, producing a magnification of 132 x. The deviation from perfect isometry of the strongest contraction was less than 2.5%.

Stimulation could be achieved by two methods: conventional

Ag-AgCl wire electrodes or massive stimulation by a homogeneous electrical field parallel to the fibers. The bar electrodes were 1 cm apart and were located near the distal end of the muscle, which passed between them. The homogeneous electrical field was set up by two platinum rings fitting snugly in the plastic cylinder above and below the preparation. Hereafter they will be referred to as M(assive) L(ongitudinal) F(ield) electrodes.

Rectangular shocks, variable in both intensity and duration, generated by a Grass model S 4 stimulator, were used for excitation. The shocks had a rise time of 2.5 micro-seconds and a fall time of 1.0 microsecond. The use of the M. L. F. electrodes decreased the resistance between the electrodes to a value around 400 ohm. This situation imposed, for the shocks of longer durations, a heavier current requirement on the stimulator than it could deliver, which resulted in a distortion of the pulse. The output of the stimulator was, therefore, fed into a cathode follower with an output impedance which matched that of the bath. The cathode follower attenuated the voltage by a factor of .567, the input voltage range was, however, large enough to compensate for this. This arrangement guarantees a uniform current density throughout the pulse and enables one to use the field strength (Volts/cm) as a measure of the stimulus, which makes comparisons between experiments with different interelectrode distance more meaningful.

B. Experimental Procedures

1. Stimulation

Before good measurements could be made it was important to find ways to achieve maximal activation of the contractile system, so that the observed effects were true reflections of the action of CO_2 on the steady state tension uncontaminated by damage to or failure of the excitable system. These considerations led to the introduction and use of massive electrodes. The necessity for such a method of stimulation became clear from early experiments on the frog sartorius which were performed with stimulation by conventional Ag-AgCl wire electrodes. With these electrodes it was found that the increasing stimulus strength needed for the compensation of decreases in excitability produced by increasing CO_2 percentages resulted in damage to the fibers adjacent to the electrodes.

The electrodes adopted are fashioned after those described by Csapo and Goodall (1954), and consist of two platinum rings, one at either end of the muscle. The rectangular stimuli are transmitted to the muscle by the longitudinal electrical field which is produced between the electrodes when square waves are applied to them. This system is a variation of the original massive electrode assembly introduced by Brown and Sichel (1936) in their studies on single muscle fibers. The latter authors applied a field at a 90^{o} angle to the fibers, rather than parallel, by applying voltages to two strips of silver of the same length as the fiber which was

placed between them. The assembly rested on a glass microscope slide and was covered by a cover slip. This arrangement produced a small chamber in which a strong electrical field with a homogeneous current distribution could easily be produced. With these electrodes Brown and Sichel established that stimulation could be achieved without the intervention of a conducted action potential.

In the case of whole muscles, especially of the length of the retractor penis (up to 7 cm), the resistance of such an electrode system would become very low and impose extreme current requirements on the electronic gear producing the stimuli. Furthermore, the homogeneity of the field would depend greatly on the perfect parallelism between the two long electrodes. The massive longitudinal field (M. L. F.) electrodes were, therefore, adopted for the present study. The electrode resistance was yet low compared to the output resistance of the stimulator and consequently the shocks were fed into a cathode follower circuit before being applied to the electrodes.

Massive stimulation enables one to avoid some of the difficulties inherent in the use of stigmatic electrodes in Ringer's solution. Such as the occurrence of damage to some fibers when the stimulus strength has to be raised to compensate for loss of excitability in the presence of high CO_2 partial pressures. When using wire electrodes, with the muscle in Ringer's solution, the current does not flow entirely through the muscle, but a large part will

be conducted by the solution. This current distribution will especially lower the stimulus strength in the interior part of the tissues with the result that a shock increased in strength so as to excite the interior fibers will be damaging to those directly in contact with the electrodes. Another consideration, speaking in favor of the adoption of massive stimulation, was the supposition that in case of conduction failure due to chemical manipulations it might be possible to effect proper stimulation by an increase of the strength of the stimulus until the fibers are excited along their entire length.

As an experimental verification of the last notion a muscle was exposed to procaine in a concentration well known to abolish the conduction of action potentials. Using the stigmatic electrodes the tension development in this muscle was approximately 10% of that before the addition of procaine. However, with the M. L. F. electrodes a tension equal to 70% of the control could still be achieved. From this simple experiment as well as from a study of the literature (Brown and Sichel, 1936; Csapo and Goodall, 1954) it is clear that massive stimulation can indeed excite the muscle locally along nearly its entire length and thereby achieve stimulation without conduction.

2. Tension

The great majority of the experiments involved equilibration of the muscles with Ringer's solutions containing different amounts of bicarbonate and variation of the CO_2 partial pressure with each

of these. (A few experiments are also reported in which phosphate buffer was used and where the pH was varied either by CO_2 or by changes in the ratio of the phosphate ions.)

The muscles were excised and placed for a minimum of two hours at 20° C in the desired bicarbonate Ringer's solution equilibrated with a CO_2 - O_2 mixture to produce a pH of 7.15 to 7.3. (Over this range the tension is practically constant and can be used for reference.) For the remainder of the run this constituted the control solution in which recovery from subsequent treatment was always allowed to take place. For the data on tension development, the muscles were tetanized maximally at intervals of 15 minutes until the tension developed in successive tests did not vary more than 2%. The solution was then replaced by one of the same composition, but equilibrated with a higher percentage of CO_2 and aerated with this gas mixture. Although the maximal and final effect is reached after 6 to 8 minutes a standard equilibration time of 10 minutes was chosen for 20° C, while 15 minutes was allowed at temperatures below 10° C. The response in question was then recorded and the muscle was immediately returned to the reference solution. It is especially important to allow recovery to take place in the reference solution since in the acid solution recovery is incomplete and the muscle deteriorates rapidly. Twenty-five minutes was allowed for recovery, a control tetanus was recorded, and fifteen minutes later the procedure was repeated with a dif-

ferent CO_2 percentage. In this fashion at least four different volumes per cent of CO_2 were used at one bicarbonate level.

The solution was then exchanged for one with a new HCO_3^- concentration and a new CO_2 percentage, again producing a pH of approximately 7.2. After two hours for recovery and re-equilibration the above experimental cycle was repeated. Usually two and occasionally three bicarbonate concentrations could be tested before the muscle began to deteriorate and the tension data began to show variations of more than 3% at which point the preparation was discarded. The major factor in deterioration is the long exposure to highly acid solutions. It was always observed that recovery after tetanus was greatly impaired if the muscle was left in the acid solution, extensive fatigue occurring following two or three tetani. This agrees with observations that aerobic recovery is greatly retarded at high CO_2 percentages (D. K. Hill, 1940).

Before good measurements could be made, the effect of increased CO_2 on the changing characteristics of effective stimulation for the production of tetanic contractions had to be studied. The changes in excitability of the tissue are considerable but can be overcome by the use of supramaximal stimuli. Early during the investigation it was also found that the increased CO_2 pressures produced a decrease in the frequency of stimulation necessary for a maximal, tetanic contraction. This "optimal" frequency is of crucial importance to the correct performance of the experiments, as both

too high and too low a rate result in a lowering of the tension. In the subsequent experiments the frequency was, therefore, always diminished in proportion to the increase in the pCO_2. During the tetanic contraction itself the frequency was quickly increased and decreased about 10 per cent and the effect on the maximal tension was noted. If it was found that the anticipated, optimal frequency was considerably different from the one found in this manner, exposure to that pCO_2 was repeated after the usual recovery time in the reference solution.

The retractor penis muscle was found to be ideally suited to this procedure as the maximal tetanic tension is maintained for several seconds. The frog sartorius, however, retains a really stable tension level for less than a second only. This fact greatly increases the number of trials at each pCO_2, because the optimal frequency could only be checked by several exposures to the same pCO_2. This, in turn, limits the amount of useful information that can be gathered for one preparation. The much smaller variability of the results for the retractor penis as compared with those for the sartorius are due in large part to this difference between the two preparations.

The effect of increased CO_2 pressure on the frequency for maximal tetanic tension differed when using just maximal stimuli and when employing stimuli four to five times stronger than the maximal voltage, i.e., "supramaximal" shocks. In all experiments the optimal frequency was determined as outlined above. In the

reference solution the frequency for barely maximal voltage and that for supramaximal stimuli were found to be within 2% of each other. After ten minutes at an increased CO_2 tension the voltage for maximal twitch tension was determined. Tetanic stimulation employing this maximal voltage was then given and the new, lower optimal frequence found. In this fashion it was found that this optimal frequency decreases with increasing CO_2 concentration. When tetani were produced in the acidified solution using stimuli four to five times stronger than the maximal voltage a smaller decrease was found with increasing CO_2 tension. The difference between the two frequencies was sometimes as high as 20%.

This dependence of the optimal frequency on the strength of the stimulation precluded the development of an accurate expression from which the correct optimal frequency could be predicted for a certain CO_2 partial pressure. It was, therefore, necessary to make a check during each tetanus, as described above.

3. Excitability

Strength-duration curves were determined in the standard fashion for both types of electrodes. The stimuli were rectangular shocks, from the Grass-stimulator, whose intensity were measured with a voltmeter from the D. C. output of the cathode follower. As indication of effective stimulation the smallest visible twitch was used. First the rheobasic field strength, E_o, was determined, and subsequently the threshold for decreasing durations. At the end

of the run the curve was quickly plotted, after which the rheobase and several other point were checked. If these checks agreed within 5% of the original values some additional points were supplemented in strategic areas, such as near a break in the curve. If the checked values did not agree within 5% of the original data, the muscle was rested for a period of one-half to one hour and the experiment repeated. If the excitability was then still found to be fluctuating, the preparation was discarded.

C. Analytical Methods

In this section the method of analysis of the inhibition is discussed and the method of calculation of an index to the excitability is presented.

1. Inhibition analysis

As a method of analysis of the inhibitory action of CO_2 the logarithmic expression of the fractional analysis of Fisher and Ohnell (1940) was chosen (see also Johnson, Eyring and Polissar, 1954). This analysis starts out with the reasonable assumption that an equilibrium exists between the enzyme and the inhibitor according to the following scheme

$$E + nI \rightleftharpoons E(I)_n \qquad (1)$$

where

E = the free enzyme
I = the inhibitor
$E(I)_n$ = the enzyme-inhibitor complex
n = the number of molecules of I combining with E to give $E(I)_n$

The amount of E left over is then thought to determine the speed of the reaction as $E(I)_n$ is considered to be inactive enzymatically. For this equilibrium the constant, K, is then

$$K = \frac{[E][I]^n}{E(I)_n} \qquad (2)$$

If we now consider the fraction of remaining activity, y, to be directly related to the concentration of E, and the fraction of lost activity, (1-y), to the concentration of $E(I)_n$, then we can substitute and rearrange equation (2) to obtain

$$\frac{y}{1-y} = K\,[I]^{-n} \qquad (3)$$

or logarithmically

$$\log \frac{y}{1-y} = \log K - n \log [I] \qquad (4)$$

From this last expression it is obvious that plotting log (y/1-y) vs. the log of the inhibitor concentration should produce a straight line with a negative slope equal to the number of inhibitor molecules that combine with the enzyme.

This analysis was applied to the inhibition of tension and excitability by CO_2 with some measure of success. This fact should not, however, be interpreted to imply that the action of CO_2 proceeds according to the very simple scheme above. An interpretation on the basis of the occurrence of the inhibition at any later point in the reaction (activation, contraction, etc.) is not excluded. The

present analysis should rather be considered as a general method for the presentation of the action of an inhibitor and for the determination of the equilibrium constant and the stoichiometric factor for the combination of the inhibitor with its target.

2. Choice and calculation of an index to excitability

When the CO_2 content of the solution bathing a muscle is increased, the threshold for both stigmatic and M. L. F. electrodes rises and a shock of any given voltage has to flow longer to produce a response. In order to investigate the phenomenon, it was decided to map out the strength-duration curve (S. D. C.) for effective stimulation and to find a convenient parameter of it for comparison at various CO_2 tensions.

A double logarithmic method of representing the data was first advocated by Rushton (1931). It has the advantage of emphasizing the region of the shorter durations and thereby accentuating the "break" in the curves denoting the change from one to another kind of stimulation. It is also useful in the determination of the muscle chronaxie if its value is obscured by the nerve branch. The double logarithmic scale suppresses the curvature of the line and facilitates the extrapolation of the muscle branch to the shorter durations. But, except for the chronaxie, a rather arbitrary parameter, it does not provide any other useful information of a quantitative nature.

In order to compare different S. D. C.'s on a quantitative basis

some manner of an index of the relation should be found. Several attempts have been made to represent the S. D. C. in mathematical formulation. These attempts have mostly been made for the relations found in nerve and the results have been carried over to the muscle, usually without too much of a critical study. Of the several attempts the one generally considered most successful for nerve is the equation developed by Hill (1936) for rectangular pulses:

$$\frac{R}{V} = 1 - e^{-t/K} \qquad (5)$$

in which

R = Rheobase (i.e., effective voltage at infinite duration)
V = Voltage effective for a particular duration
t = Duration of stimulus
K = The dynamic time constant of the membrane with the dimension of time

Another equation, developed by Blair (1932) and applicable to many nerve S. D. C.'s is:

$$\frac{V-R}{V} = \frac{C-1}{C} e^{-at} + \frac{1}{C} e^{-kt} \qquad (6)$$

in which the same symbols have the same meaning in addition to which

a and k = dynamic time constants of the dimensions of time^{-1}
C = a dimensionless constant

Without the use of neuromuscular blocking agents it is possible to obtain data on the excitation of the muscle membrane directly,

which suffice for the calculation of Hill's K, and Blair's k and C. These indices can, however, not be determined from Rushton's plot and Blair's method for representing the S. D. C.'s was adopted.

Starting with equation (6), Blair points out that a is much larger than k, around 10 x. Therefore, the last term, $1/Ce^{-kt}$, is dominant at longer durations and the equation simplifies to

$$\frac{V-R}{V} = \frac{1}{C} e^{-kt} \qquad (7)$$

Taking logarithms

$$\ln \frac{V-R}{V} = -kt + \ln \frac{1}{C}$$

and rearranging

$$\ln \frac{V}{V-R} = kt - \ln c$$

or

$$\log \frac{V}{V-R} = \frac{k}{2.303} t - \log c \qquad (8)$$

he arrives at an equation that is adapted to graphic representation and analysis of the S. D. C. data at longer durations.

If one now plots log (V/V-R) vs. t in seconds one should obtain a curve consisting of two linear portions connected by a curved part. The straight line at the longer durations corresponds to the region where (7) holds. The intercept of this line with the ordinate is C, while the slope is k/2.303.

This method of plotting S. D. C. data provides an easy way of calculating the two constants k and C. The only drawback is the

fact that the nerve branch of the S. D. C. obliterates the muscle branch at the shorter durations, and that constant a can not be determined without the use of neuromuscular blocking agents. The advantage of this method of plotting is the fact that it aids in the visualization of the difference between Hill's and Blair's equations, (5) and (6) respectively, and provides a sensitive test for the validity of the two expressions. In Hill's expression C is tacitly assumed to be unity, in which case equations (5) and (6) are identical. This can easily be shown by setting C = 1 in (6). It is then obvious that Blair's k is the reciprocal of Hill's K, as shown by reducing (7) to

$$\frac{V-R}{V} = e^{-kt}$$

$$\frac{R}{V} = 1 - e^{-kt} = 1 - e^{-t/K} \qquad (9)$$

i.e., Hill's expression. Rearranging and taking logarithms we get:

$$\log \frac{V}{V-R} = \frac{k}{2.303} t \qquad (10)$$

If this relation is correct a plot of log V/V-r vs. t should be linear over its entire range and intersect the vertical axis at the origin (i.e., C = 1).

CHAPTER IV

RESULTS

A. Tetanus Tension

In Fig. 1 the variation of the tetanic tension with the temperature is shown for two retractor penis muscles over the range from 0 to 20° C. One of these muscles was excised in the summer from a turtle acclimatized to 20 - 25° C, the other from a winter turtle kept at 5 - 10° C. Similar curves and similar seasonal shifts are found for frog muscles.

Figure 1 shows that in the muscles of cold-adapted animals a plateau level of tension is reached at about 12° C, while in the warm-adapted individuals this does not occur until 17° C. There occurs, therefore, a seasonal shift in the temperature at which the muscle is "totally activated" (Brown, 1957) during a tetanic contraction. To guarantee the presence of complete activation when studying the effects of CO_2 on the tension developen in this state, it was decided to make these studies at 20° C, where in both winter and summer the plateau has been reached. The investigation of the CO_2 effect in a not "totally activated" region will be performed at 0° where the failure to achieve maximal activation is most marked.

The lower curves in Fig. 1 are the responses to 80% CO_2 of two cold adapted retractor penis muscles (equilibrated with 15 mM $NaHCO_3$).[3]

3. Although the CO_2 solubility is almost 2 times larger at 0° than at 20° C, this is offset by an increase of the pK_1' from 6.13 at 20° to 6.38 at 0° C. The result is that at equilibrium the pH values differ by only 0.03 pH units.

Figure 1

TETANIC TENSION AS A FUNCTION OF TEMPERATURE

IN THE TURTLE'S RETRACTOR PENIS MUSCLE

Abscissa: temperature in ^{o}C

Ordinate: tension as a fraction of the tension at 20^{o} C

Left curve winter turtle, middle curve summer turtle, right curves winter turtle muscle in 80% CO.

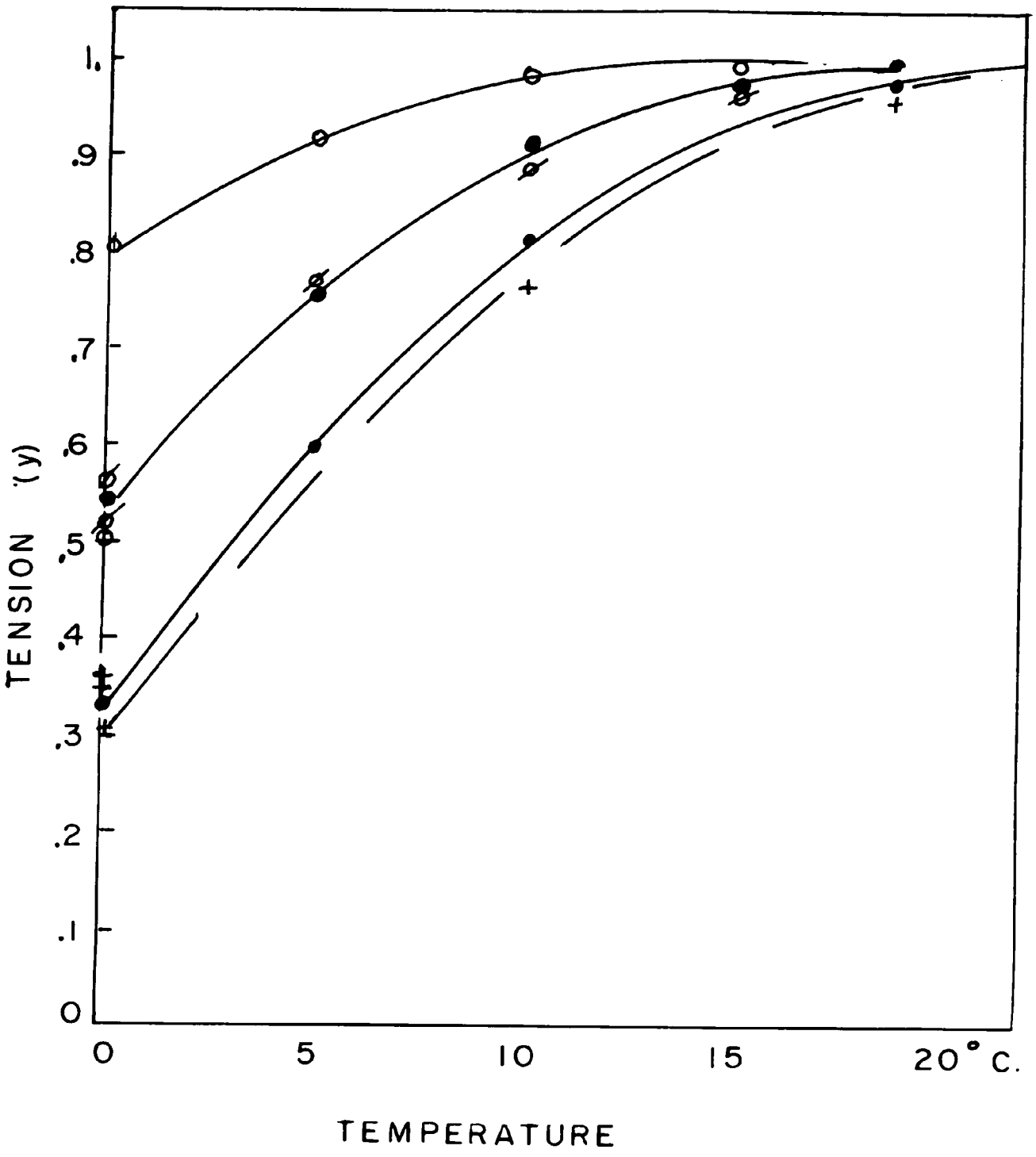


Figure 1

The inhibition is shown to be enhanced at the lower temperatures, which produces the appearance of a shift from low to high temperature acclimatization. Because recovery is much faster at 20° C and so permits a faster experimental schedule, it was decided that the bulk of the experiments would be carried out there. Afterwards, the proof of the mode of action of the CO_2, and experience in the analysis of the data could then be carried over to the work at the lower temperature.

1. The mode of action of CO_2

The results of three typical experiments at 20° C on two muscles are shown in Fig. 2 where the tetanic tension in per cent of that at the reference pH is plotted as a function of increasing pCO_2 (expressed as volumes per cent of CO_2 - O_2 mixtures). The curves represent runs at different bicarbonate concentrations with which the muscles were equilibrated beforehand. The runs at .5 and 5.0 mM were performed on one muscle, the run at 1.5 mM on the other.

It is immediately obvious that the CO_2 has an inhibiting action on the tension which is in some way related to the bicarbonate concentration with which the muscle was equilibrated. This effect could be mediated in three possible ways:

1. By changes in the external pH
2. By a specific effect of physically dissolved CO_2 or H_2CO_3
3. By changes in the intracellular pH

Figure 2

THE EFFECT OF CO_2 ON THE TETANUS

TENSION AS A FUNCTION OF CO_2 PARTIAL PRESSURE

Abscissa: logarithm of the pCO_2 expressed as volume per cent

Ordinate: tension as % of the value in the reference solution

Retractor penis, 20° C. Curves from left to right for 0.5, 1.5, 3.5 and 5.0 mM of bicarbonate in the Ringer's

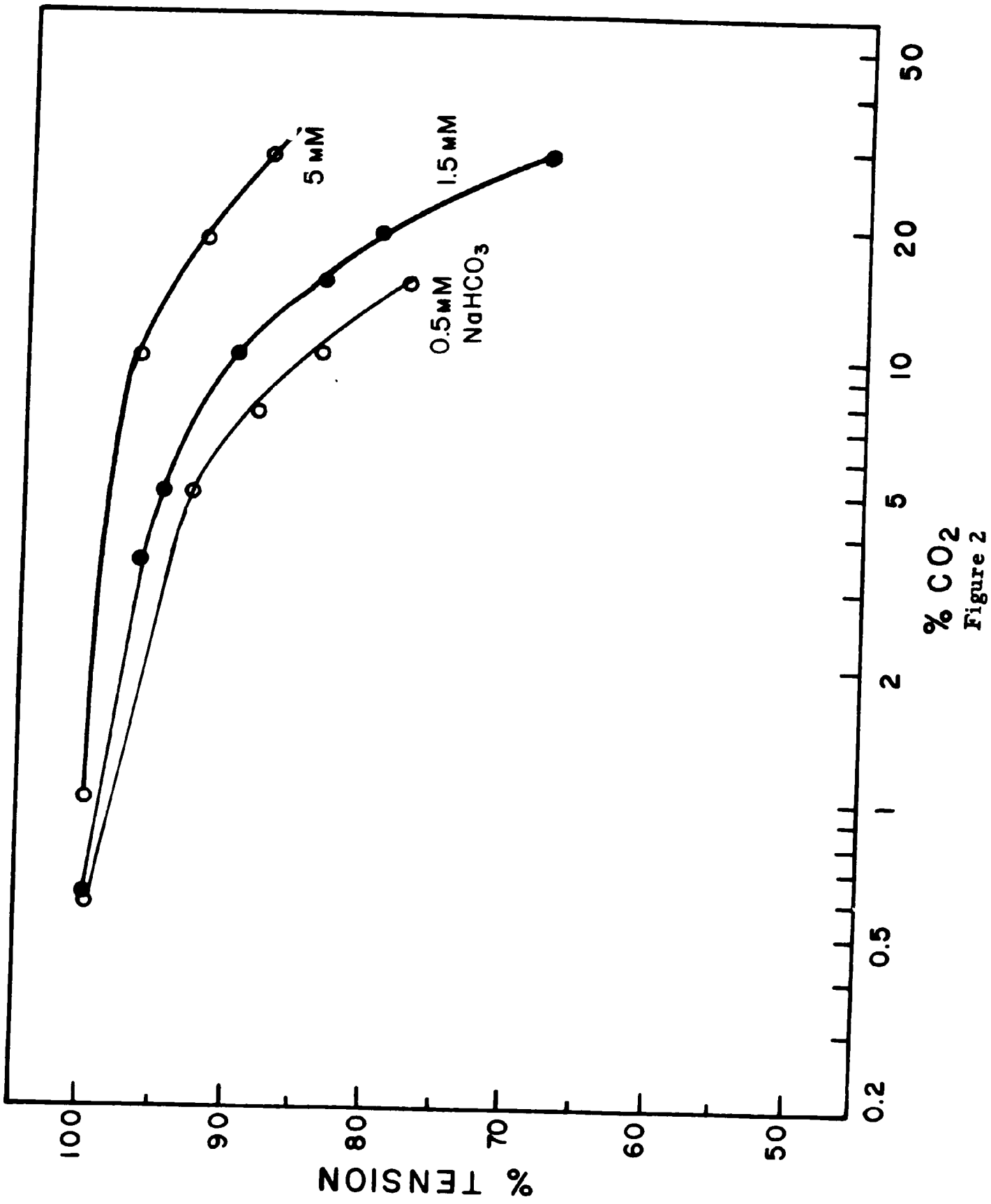

% TENSION
100
90
80
70
60
50
0.2
0.5
1
2
5
10
20
50
% CO_2
5 mM
1.5 mM
0.5 mM $NaHCO_3$

Figure 2

The possibility that the first mechanism is responsible for our results was studied by a series of experiments on the effect of approximately equal pH values produced either by CO_2 or by changes in the ratio of HPO_4^- and $H_2PO_4^-$ in a Ringer's solution buffered by phosphate. Table 2 lists these experiments, for which both turtle's retractor penis muscles and frog's sartorii were used. The first column lists the type of buffer used, which in all cases was adjusted to pH 7.2 to 7.3. Column 2 represents the changed pH of the solution to which the muscles were exposed for 15 minutes before the reading was taken. The per cent change between the tetanic tension at the reference pH (always set equal to 100%) and at the new pH is shown in column 3, which lists the average for the determinations made, whose number is indicated by the figure in the next column. The last column shows the method by which the pH change was brought about.

A study of the table reveals that there is no correlation between the external pH and the amount of change in tetanic tension, neither between the phosphate buffer experiments among themselves, nor between these and the ones with bicarbonate. Comparison of lines 2 and 3, for instance, shows that two approximately equal pH changes brought about by a change in the phosphate buffer or by 100% CO_2 have a tenfold different effect. (The possibility that the feeble contractions of the muscles in 100% CO_2 were due to the anoxia can be ruled out, as it is a well proven fact that muscles can contract

Table 2

THE EFFECT OF EXTERNAL pH CHANGES ON TETANIC TENSION

Turtle Retractor Penis Muscles				
Buffer	pH ext	Average % F_o	# of muscles	Method of changing the pH
15 mM Phosphate	6.20	-3.2	2	change in the ratio of $H_2PO_4^-$ to $HPO_4^=$
" "	5.45	-5.4	3	" "
" "	5.52	-58.3	3	100% CO_2
2 mM "	5.35	-5.3	3	change in the ratio of $H_2PO_4^-$ to $HPO_4^=$
" "	5.32	-25.7	4	20% CO_2
15 mM $NaHCO_3$	6.25	-4.8	2	30% CO_2
" "	5.73	-16.0	6	100% CO_2
5 mM "	5.45	-30.6	2	100% CO_2
3.5 mM "	5.38	-37.0	5	100% CO_2
2.5 mM "	5.48	-20.1	2	30% CO_2

Table 2 (Continued)

THE EFFECT OF EXTERNAL pH CHANGES ON TETANIC TENSION

Frog Sartorius Muscles				
Buffer	pH ext	Average % F_o	# of muscles	Method of changing the pH
5 mM Phosphate	5.83	-7.4	4 3	change in the ratio of $H_2PO_4^-$ to $HPO_4^=$
" "	5.95	-19.8	3	10% CO_2
1 mM "	5.79	-2.5	3	change in the ratio of $H_2PO_4^-$ and $HPO_4^=$
" "	5.84	-14.1	2	5% CO_2
15 mM $NaHCO_3$	6.89	-2.2	1	7% CO_2
" "	5.73	-12.2	1	10% CO_2
5 mM "	6.45	-2.2	1	5% CO_2
" "	6.23	-4.2	4	10% CO_2
3.5 mM "	5.78	-12.7	5	20% CO_2
" "	5.38	-37.1	1	100% CO_2
2.5 mM "	6.10	-7.2	3	7% CO_2
" "	5.77	-10.2	1	15% CO_2
" "	5.48	-19.7	2	30% CO_2
1.0 mM "	5.51	-25.3	1	20% CO_2

anaerobically without great changes in their performance). Scrutiny of the other experiments reveals the same lack of correlation, and it can be concluded that the CO_2 does not affect the muscle through changes in the acidity of the external fluid.

The second possible mode of action, that of a specific effect of the dissolved CO_2, or the H_2CO_3 produced from it, can be eliminated by consideration of the experiments shown in Fig. 2. Here we see that equal partial pressures of CO_2 have quite different effects at different bicarbonate levels. As the physically dissolved CO_2 is linearly related to the pCO_2 and as the H_2CO_3 concentration is directly dependent on the dissolved CO_2, these two cannot be the agents in the reduction of the tension.

The action of CO_2 must, therefore, be assigned to its effects on the intracellular pH.

2. The relation between pCO_2 and tension

The relation of increased partial pressures of CO_2 to the tetanic tension was shown in Fig. 2, while some other figures were presented in Table I. During the initial experiments it was quickly found that all the data for the frog muscle and those for the turtle muscle at high CO_2 pressures (75%) exhibited a large amount of variability. In the case of the

frog muscle this might be accounted for by the difficulties in finding the exact optimal frequency of stimulation for each pCO_2. In the retractor penis muscle the tetanus tension is stable for several seconds, allowing one to find the exact optimal frequency and therefore the true maximal tension. In the frog sartorius, however, this is not the case. Here the tetanus tension is stable for less than one second so that the correct, optimal frequency has to be chosen beforehand (see also "Materials and Methods"). It is thought that the great variations in the results with the sartorius may largely be due to this factor. The greater variability of the retractor penis muscles at the higher CO_2 pressures can, however, not be explained in this fashion. An explanation might be sought, however, in some other cellular effect of the low pH or a secondary shift of ions from or to the muscle. In view of these two difficulties, it was decided to limit the investigation to the behavior of the retractor penis muscle of the turtle at relatively low CO_2 pressure, i.e., 30% or lower. The results at 20° shown in Fig. 2 were run under these limiting conditions.

The action of CO_2 must be assigned to its intracellular acid properties, as was shown in the preceding section. The system provides us, therefore, with a tool for the study of the contractile machinery as a function of intracellular pH. As it is difficult, however, to draw reliable conclusions about the

behavior of the system from the limited range of CO_2 percentages dictated by the experimental procedure, it was necessary to make a large number of measurements at the four bicarbonate concentrations used. Because of the uncertainty of the extrapolation of the results at one bicarbonate in one muscle to another HCO_3^- concentration in another, attempts were made to establish the relation for each preparation at as many bicarbonate concentrations as possible. It turned out that usually two but occasionally three runs at different base concentrations could be made before irreversible effects set in.

In Fig. 3 the data for 31 runs on 17 muscles at four different HCO_3^- concentrations are presented in a mass plot. As a method of presentation the logarithmic expression of the fractional analysis of Fisher and Ohnell (1940) was chosen. The data are plotted as $y/1-y$ = (fractional tension remaining)/(fractional tension inactive) vs. the logarithm of the CO_2 partial pressure. The vertical bars delimit the spread of the results at one pCO_2, while the circle indicates the arithmetical average. The straight lines were drawn by the method of least squares through the circles belonging to each HCO_3^- concentration. The slopes of the lines are -1.03, -0.94, -0.95, and -1.05 for .5, 1.5, 3.5 and 5.0 mM bicarbonate respectively. The relation between CO_2 and tension is, therefore, expressed by $\log y/1-y = \log K - n \log [CO_2]$ where the value of K varies with the HCO_3^-

Figure 3

FISHER - OHNELL PLOT OF THE CO_2 EFFECT ON TENSION
(MASS PLOT)

Abscissa: logarithm of the pCO_2

Ordinate: logarithm of the ratio of the uninhibited to the inhibited fractions

Retractor penis, 20° C. Curves from left to right for 0.5, 1.5, 3.5 and 5.0 mM of bicarbonate in the Ringer's. Mass plot for 17 muscles, 31 runs. The vertical bars indicate the range of the determinations, whose number is written below.

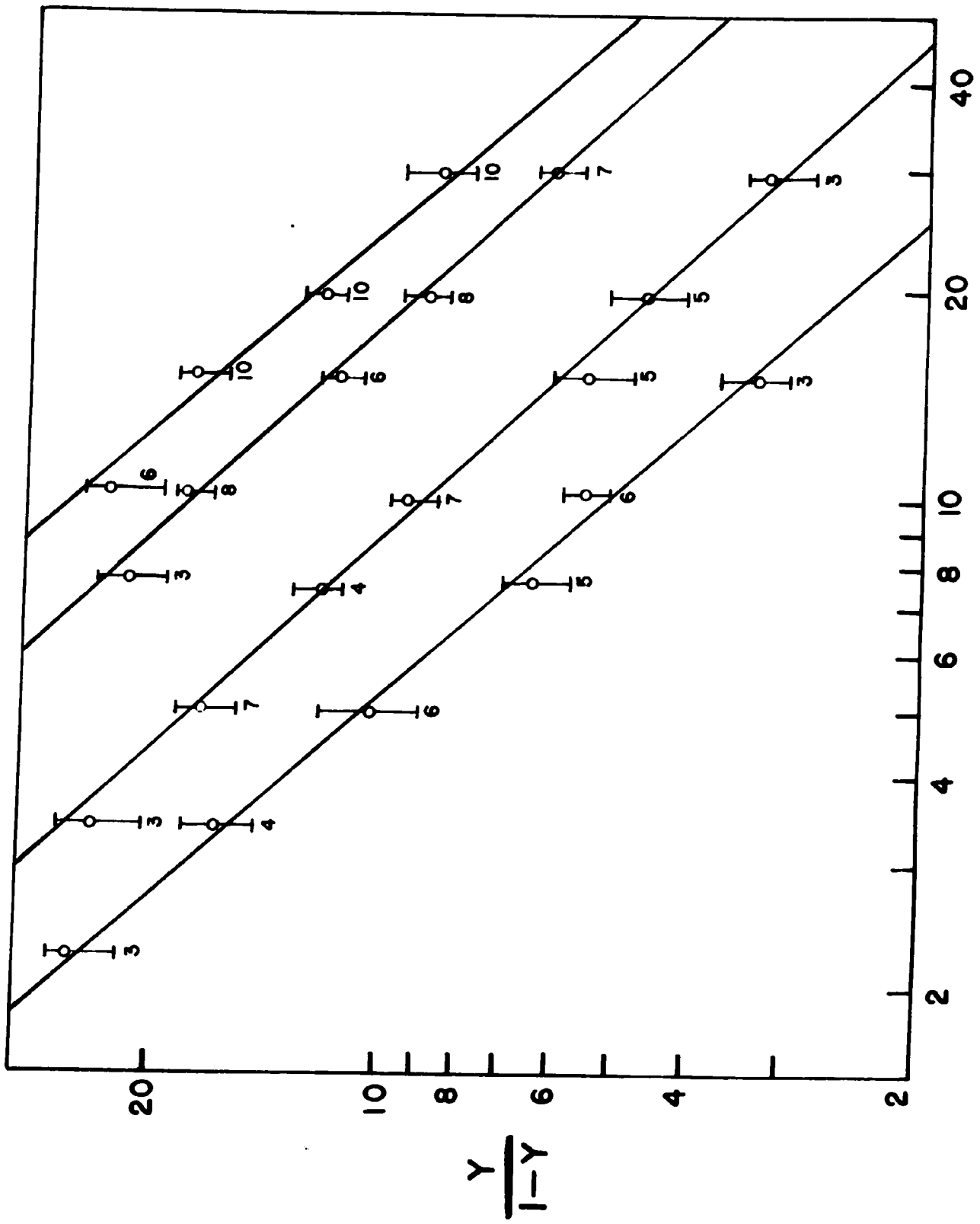


Figure 3

concentration.

The fit of the data to a straight line relation is satisfactory. The spread of the points tends to be slightly larger at the higher y/1-y values, which is to be expected because a certain experimental error is much magnified by this analysis when the inhibition is small.

The relation between tension inhibition and the CO_2 partial pressure is apparently dependent on the amount of bicarbonate in the Ringer's solution. In the previous section it was shown that the CO_2 produced its effect by changing the intracellular H - ion concentration. The shifts of the lines of Fig. 3 to higher CO_2 tensions with increasing bicarbonate concentrations, shows that the effect of the CO_2 is diminished by increased bicarbonate. In other words, the HCO_3^- - CO_2 relation is that of a buffering action.

The quantitative relations of this buffering action can now be analyzed. On the basis that the action of the CO_2 is through its effect on the intracellular pH, we can consider that at an equal amount of inhibition the intracellular pH must have been the same. Graphically this means that in Fig. 3 equal values of (y/1-y) indicate that the particular combinations of HCO_3^- and pCO_2 that produce these values, produced the same intracellular pH. In Fig. 4 the buffering action of the HCO_3^- on the CO_2 effect is presented graphically. Taking a particular (y/1-y)

Figure 4

pCO_2 NEEDED FOR EQUAL INHIBITION AT VARIOUS BICARBONATE CONCENTRATIONS

Abscissa: mM $NaHCO_3$ in the Ringer's

Ordinate: pCO_2 necessary to give equal inhibition, i.e., constant $(y/1-y)$ value in Fig. 3

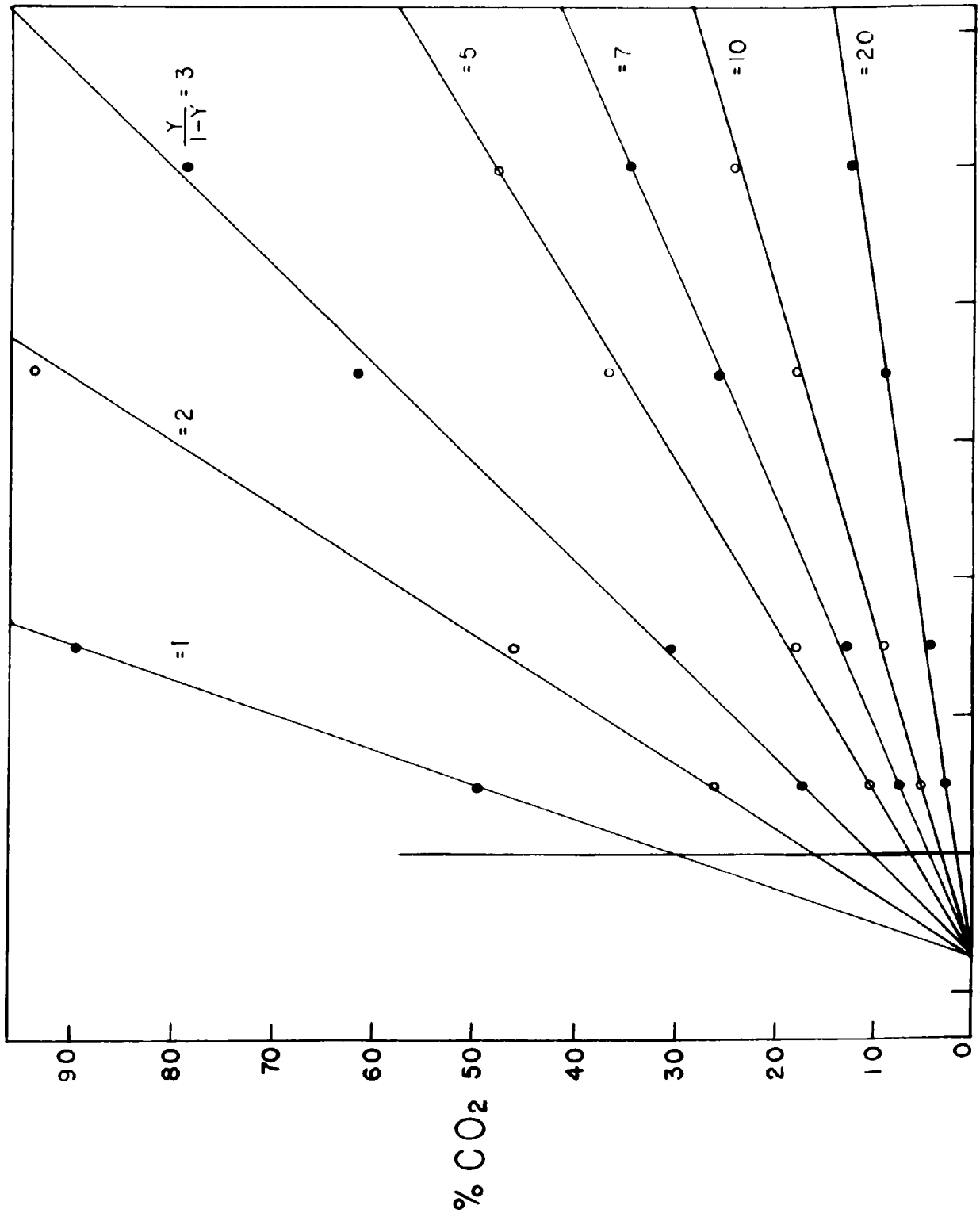

% CO_2
mM HCO_3^-
$\frac{Y}{1-Y}$ = 3
=1
=2
=5
=7
=10
=20
0
10
20
30
40
50
60
70
80
90
-1
0
1
2
3
4
5
6

Figure 4

value in Fig. 3, we read across the graph and note the pCO_2 that produced this level of inhibition for each of the four bicarbonate concentrations. These CO_2 tensions are plotted in Fig. 4 as a function of the bicarbonate concentration for the various y/1-y values noted on the graph, and straight lines are fitted through each set of points.

Since in this graph we are in effect plotting the last term of the Henderson-Hasselbalch equation:

$$pH = pK - \log \frac{base}{acid},$$

the linearity establishes that log (y/1-y) is varying as a linear function of pH and may be described by

$$\log \frac{y}{1-y} = npH$$

where n is a proportionality constant.

In Fig. 4 the straight lines upon extrapolation intersect the abscissa at a value of minus 0.76 mM $NaHCO_3$. This extra base must be considered as the buffering capacity of the other components of the cell. Smith (1926), using a different method of analysis, found a qualitatively similar relationship for isotonically contracting turtle heart tissue. He found this extra amount of base t. be equal to about 2.4 mM of bicarbonate and referred to it as the "fixed intracellular base." It is interesting to analyze the present data also according to his method in order to see if the differece in the twc values for the fixed

intracellular base, 0.76 and 2.5 mM, might be due to the difference in analytical method.

In Smith's analysis the extent of the decrease of the tetanic tension is again viewed in the light of intracellular pH changes. If we consider the per cent decrement as an index of the amount of change of intracellular pH, then the effectiveness of the CO_2 in producing such decrements should be related to the amount of intracellular base as a consequence of the mass law. In order to visualize this analysis some experiments are plotted in Fig. 5 as the percent decrease in tension as a function of the increasing pCO_2 for several HCO_3^- concentrations. Over the range of pCO_2 used the curves can be considered linear, in analogy with Smith, and they show that the higher the bicarbonate concentration, the less effective the CO_2 is in reducing the tension.

The action of CO_2 is then analyzed in terms of its "physiological effectiveness" at various bicarbonate concentrations, i.e., the pCO_2 tension decrements are used as an index to the intracellular changes produced by the carbonic acid. This is accomplished by calculating the fraction $(\Delta HCO_3^-/\Delta$ % tension) at each bicarbonate concentration. Graphically this amounts to taking the reciprocal slopes of the lines in Fig. 5. In Fig. 6 this relation is plotted as a function of the HCO_3^- concentration. A line is drawn through the three slopes derived from runs

Figure 5

SMITH ANALYSIS OF CO_2 EFFECT ON TENSION

Abscissa: pCO_2 expressed as volume per cent

Ordinate: decrement of tetanic tension expressed as % of reference value

Retractor penis, 20° C. Curves from left to right for 0.5, 1.5, 3.5 and 5.0 mM bicarbonate in the Ringer's. Curves marked B were obtained from one muscle.

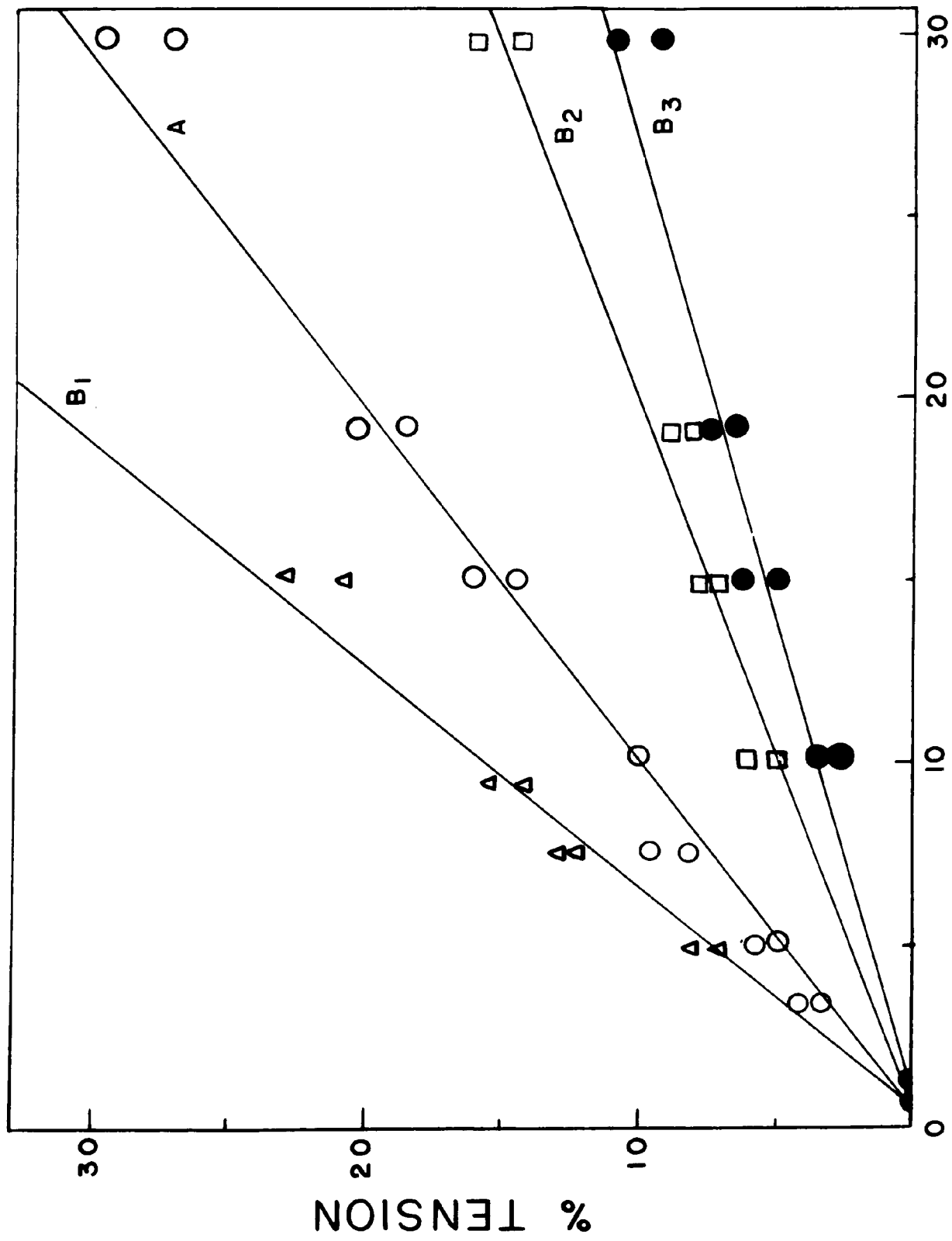


Figure 5

Figure 6

EFFECTIVENESS OF CO_2 INHIBITION AT VARIOUS BICARBONATE LEVELS

Abscissa: Ringer bicarbonate concentration (mM)

Ordinate: slope of the effect of CO_2 on the amount of inhibition

Retractor penis, 20° C.

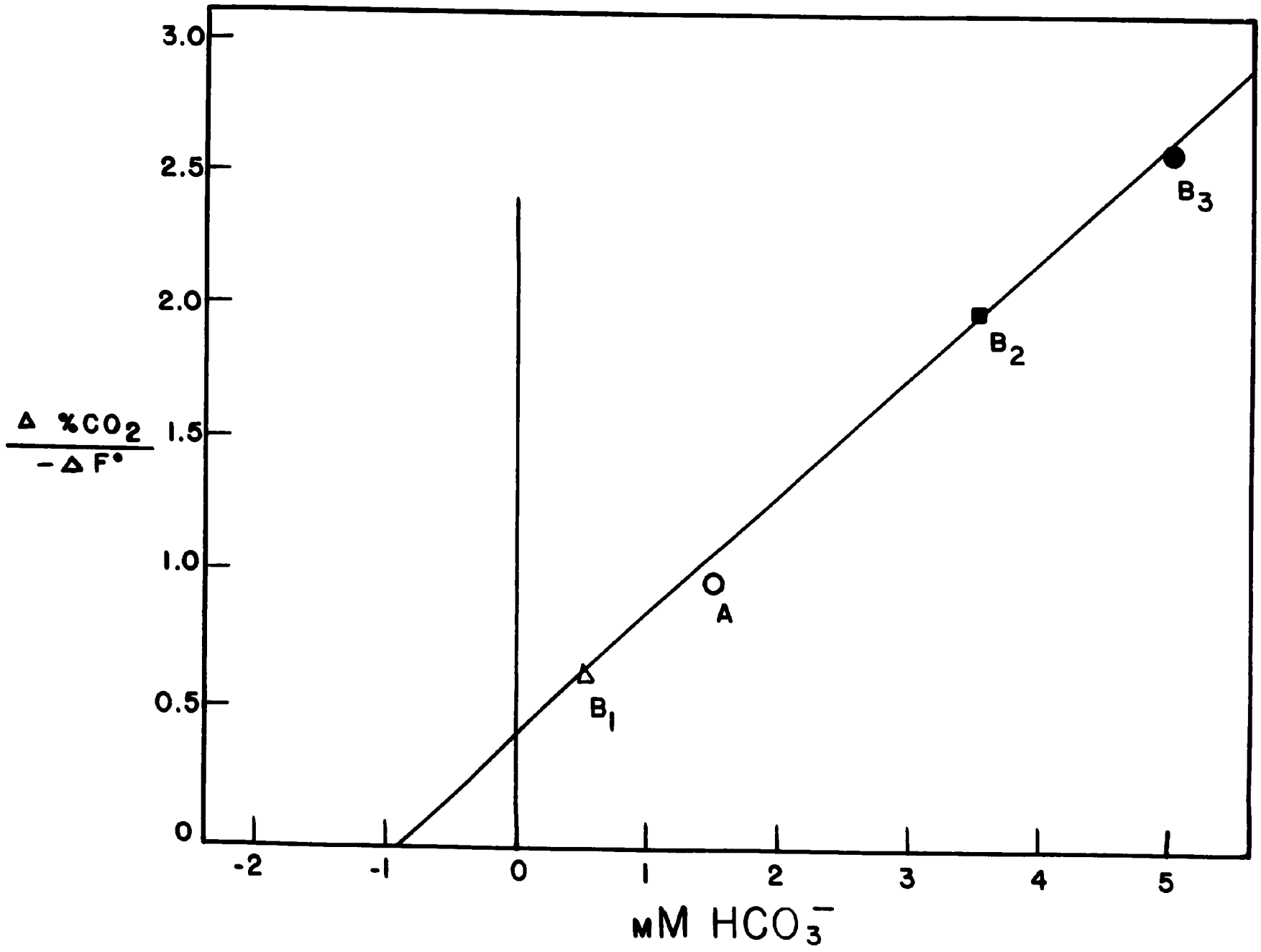


Figure 6

$B_{1,2}$ and $_3$. On the basis of the mass law, this linear relationship would be expected if the intracellular pH changed due to the increasing CO_2 concentrations in the presence of various amounts of intracellular base. Upon extrapolation the straight line intersects the base line at a distance equal to minus 0.72 mM HCO_3^-. A better quantitative analysis can be made using the data of the 31 runs at four different HCO_3^- concentrations. The values obtained for the slopes of the individual runs are plotted in Fig. 7 as a function of the HCO_3^- concentration in the Ringer's solution. The straight line is fitted to the means and is extrapolated to the base line, which it intercepts at -0.80 mM. When we plot the results with muscles, for which two or more runs were made, individually, we obtain the following series of intercepts: 0.67, 0.73, 0.77, 0.82, 0.87, 0.90, 0.91, 1.08 and 1.73 mM. These values appear to be randomly distributed around -0.85 mM, except for the last intercept. That value was obtained on a muscle using two bicarbonate concentrations, 1.5 and 3.5 mM, in close proximity on the graph. As a consequence of the proximity of these two concentrations, any error is greatly multiplied upon extrapolation. It seems, therefore, justified to ascribe this variation to experimental error and to consider the values as belonging to one population.

The slopes of the individual regression lines were also

Figure 7

EFFECTIVENESS OF CO_2 INHIBITION AT VARIOUS BICARBONATE LEVELS
(MASS PLOT)

Same as Fig. 6. The bars indicate the range of the values.

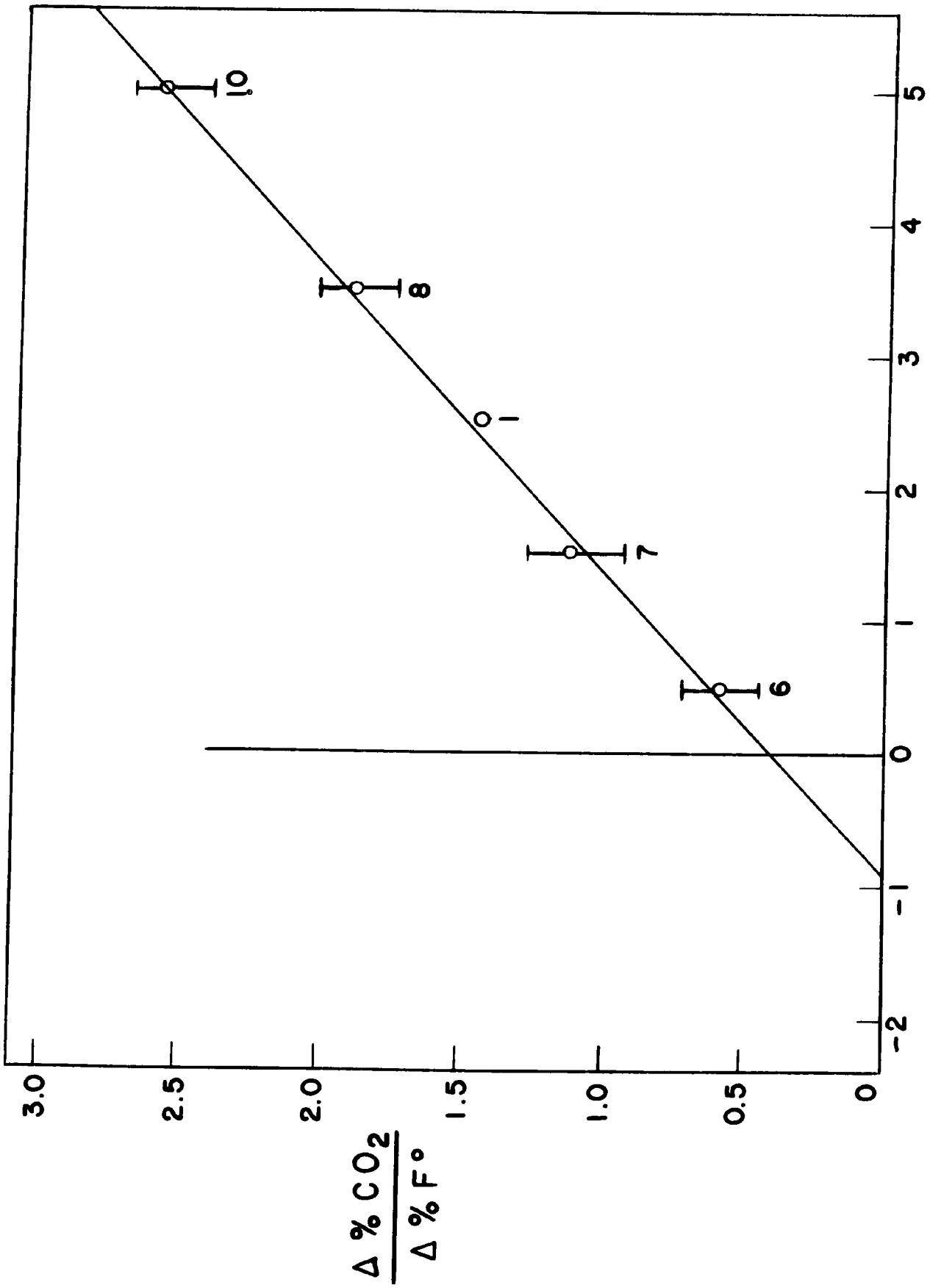

10
8
1
7
6
3.0
2.5
2.0
1.5
1.0
0.5
0
-2
-1
0
1
2
3
4
5
mM HCO_3^-
$\frac{\Delta \% CO_2}{\Delta \% F^\circ}$

Figure 7

randomly distributed around the slope for the means, with the exception of the one muscle discussed above. An indication that the slopes do not vary more than randomly between muscles is the fact that the ranges at 0.5 and 5.0 mM are approximately equal. If these slopes varied, while the intercepts remained relatively fixed, the ranges of the experimental points would increase with increasing distance from the intercept. That this is not the case is apparent from Fig. 7. It can be concluded that the spread is due to experimental error only and that the straight line which was fitted to the means reflects a fundamental propoerty of the muscles.

Smith, using the decrement of shortening of isotonically contracting cardiac muscle, found a similar relationship. He comments in his discussion on the fact that the results on one preparation could not be extrapolated to other preparations, due to a great difference in sensitivity to CO_2 between atria (i.e., the slope of the straight line in Figs. 6 and 7 was quite variable) and in the value of the extrapolated intercept. Such variability is not encountered in the isometrically contracting skeletal muscle.

The extrapolated part of the regression line of Fig. 7 intersects the base line at a value of -.080 mM of bicarbonate while the value found with the Fisher-Ohnell plot is -0.76 mM. The difference between these two values may be considered trivial.

Smith pointed out that this entity could be considered as a "fixed intracellular base." In other words, there is a constant surplus of base within the cell that has to be added to each bicarbonate concentration applied externally to represent the true variation of the tension in respect to CO_2. The chemical identity of the fixed intracellular base cannot be deduced from these experiments. It probably is not a simple entity but more likely constitutes the sum of various organic and inorganic fractions in the cell. The quantitative value of 0.76 or 0.80 mM should not be considered as an actual concentration term but more as a virtual concentration expressed in units of "buffering action" of bicarbonate buffer.

In calculation of the "intracellular pH" this factor is important. Thus, for example, when the muscle is equilibrated with 1.5 mM HCO_3, the intracellular base would appear as 2.26 mM and the intracellular pH should be calculated in respect to this figure. In later considerations of the intracellular pH this practice will be followed.

3. Experiments at lower temperatures

The effect of various CO_2 partial pressures was investigated at 0° C where the tension is reduced, supposedly by failure of complete activation. In order to avoid possible changes of intracellular ion concentrations due to changes in ion fluxes, the exposure to low temperatures was confined to the equilibra-

tion period of 15 minutes. During these experiments the muscles remained in the reference solution at 20° and the control measurements were taken at this temperature. The preparation was then exposed to the acidified solution at 0° for 15 minutes, the tetanus tension was measured and the reference solution at 20° was immediately reintroduced. For the calculation of the inhibition, the tension at 0° with the same CO_2 percentage as the reference solution at 20° was used as the 100% point, the values at other CO_2 pressures being expressed as fractions of that one.

In Fig. 8 the Fisher-Ohnell plot of tension as a function of pCO_2 is presented for seven experiments, all with 3.5 mM bicarbonate. The seven individual experiments fell into two classes; four produced a slope of 1 and three gave n=3. The data within each experiment were consistent and any particular preparation produced either one or the other response.

For one of the muscles with slope 3 at 0° the effect of CO_2 was also checked at two intermediate temperatures: 5° and 10° C. At 10° the curve was indistinguishable from the ones at 20°, while at 5° a small discrepancy was evident.

The discrepancy of the two sets of figures is large enough to exclude the possibility that the differences are due to experimental error. The explanation should rather be sought in the occurrence of differences in the material. These ex-

Figure 8

FISHER - OHNELL PLOT OF THE CO_2 EFFECT ON TENSION AT 0° C

Abscissa: log of the pCO_2

Ordinate: log of the ratio of the uninhibited to the inhibited fraction

A - 4 muscles, n = 1.13

B - 3 muscles, n = 2.82

Bicarbonate 3.5 mM throughout

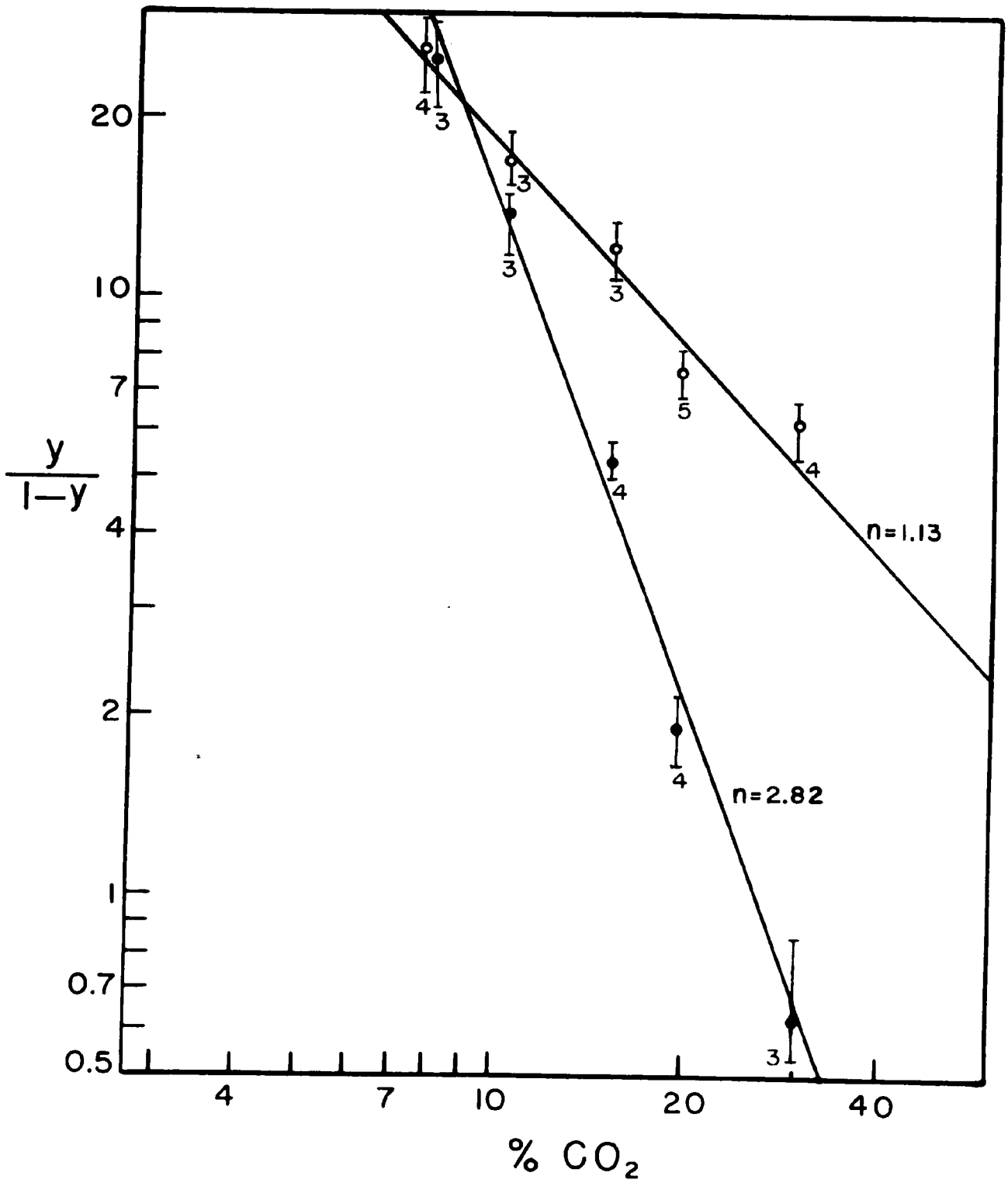


Figure 8

periments were performed in the spring and it seems highly probably that some of the turtles were already acclimatized to higher temperatures while others were not. In the cold adapted turtles the tension at 0° C has only fallen to 80% of that at 20° (see Fig. 1), i.e., the muscle was still almost totally activated. In such a muscle the effect of CO_2 on tension would still show most of the features of a totally activated muscle. These muscles would then respond to CO_2 with an inhibition of tension which proceeds with slope n = 1. Muscles of summer-acclimatized animals give only about 55% tension at 0°; in these stimulation has failed to a much greater extent in the production of full activation. The muscles that respond to pH changes with a stoichiometric factor n = 3 are likely to be in this category. This explanation is lent special support by the finding of Brown and Goodall (1956) that glycerinated fibers, in which activation is dominant, respond to pH changes with a stoichiometric factor n = 3. Additional support comes from the finding reported above, where a muscle with n = 3 at 0° is found to react with n = 1 at 10°. In a summer turtle the tension at 10° is approximately 85% of the plateau tension, i.e., the same as for the winter turtles at 0° C. From that experience it is clear that the occurrence of the relation with a slope n = 3 was found only at temperatures where the failure to achieve full activation had produced a

considerable drop in the tension development.

4. The pH vs. tension graph

In the Introduction it was concluded that a definitive value could not be assigned to the average pH of the muscle cell, while the pH of the functional site of interest is even more completely in the dark. It is yet possible to discuss the pH characteristics of the tension developing reaction in the light of the present CO_2 - bicarbonate data in reference to calculated values of the intracellular pH. Thus, if we proceed from the assumption that a true equilibrium exists between the external and the internal $NaHCO_3$ concentrations, then a quick simplification is offered us. Using a value for the "fixed intracellular" buffering capacity of 0.76 mM bicarbonate and considering it actually as bicarbonate, and not as the unknown moiety it really is, we can calculate the pH dependence of the tension from Fig. 4. In that figure the regression lines of the influence of CO_2 on tension, expressed as log (y/1-y), are plotted as a function of the external HCO_3^- concentration and the lines extrapolated to a common intercept at -0.76 mM HCO_3^-. The ordinate at zero millimolar, external bicarbonate is intersected by the regression lines at a series of pCO_2 values which should be considered as the pCO_2 values at which these particular amounts of tension inhibition would occur if an experiment were run without any bicarbonate in the Ringer's. (Ex-

perimental difficulties in the form of a lack of long range stability of the muscle under these conditions have prevented the actual performance of this experiment.) With these CO_2 partial pressures and a bicarbonate concentration of 0.76 mM the pH can be calculated via the Henderson-Hasselbalch equation for each of the (y/1-y) values. In Fig. 9 this information has been graphed as pH vs. log (y/1-y) and a straight line with a slope n = 1 fitted through the points. This graph represents the pH dependence of the isometric, tetanic tension presented in the Fisher - Ohnell plot.

In Fig. 10 the same relation has been graphed in the more usual form of pH vs. % tension (curve at left). The solid line is calculated from the straight line of Fig. 9, the broken part from its extrapolation to smaller (y/1-y) values. The individual points in the left curve of Fig. 10 are individual, experimental values found at the various bicarbonate and pCO_2 combinations for the turtle retractor penis. These were reported in Fig. 3. (Because of the great number of values for the retractor penis every 4th determination has been plotted only.) In the same manner the pH vs. activity curve is drawn for the data at 0° C for which the Fisher-Ohnell plot has a slope n = 3 (curve at right). For the calculation of the pH of the experimental points the fixed intracellular base is again added to the bicarbonate concentration in the Ringer's and the

Figure 9

THE pH vs. ACTIVITY CURVE FOR TENSION
(FISHER - OHNELL PLOT)

Abscissa: pH calculated via the Henderson Hasselbalch equation from Fig. 4

Ordinate: log of the ratio of the uninhibited to the inhibited fraction

Retractor penis, 20° C

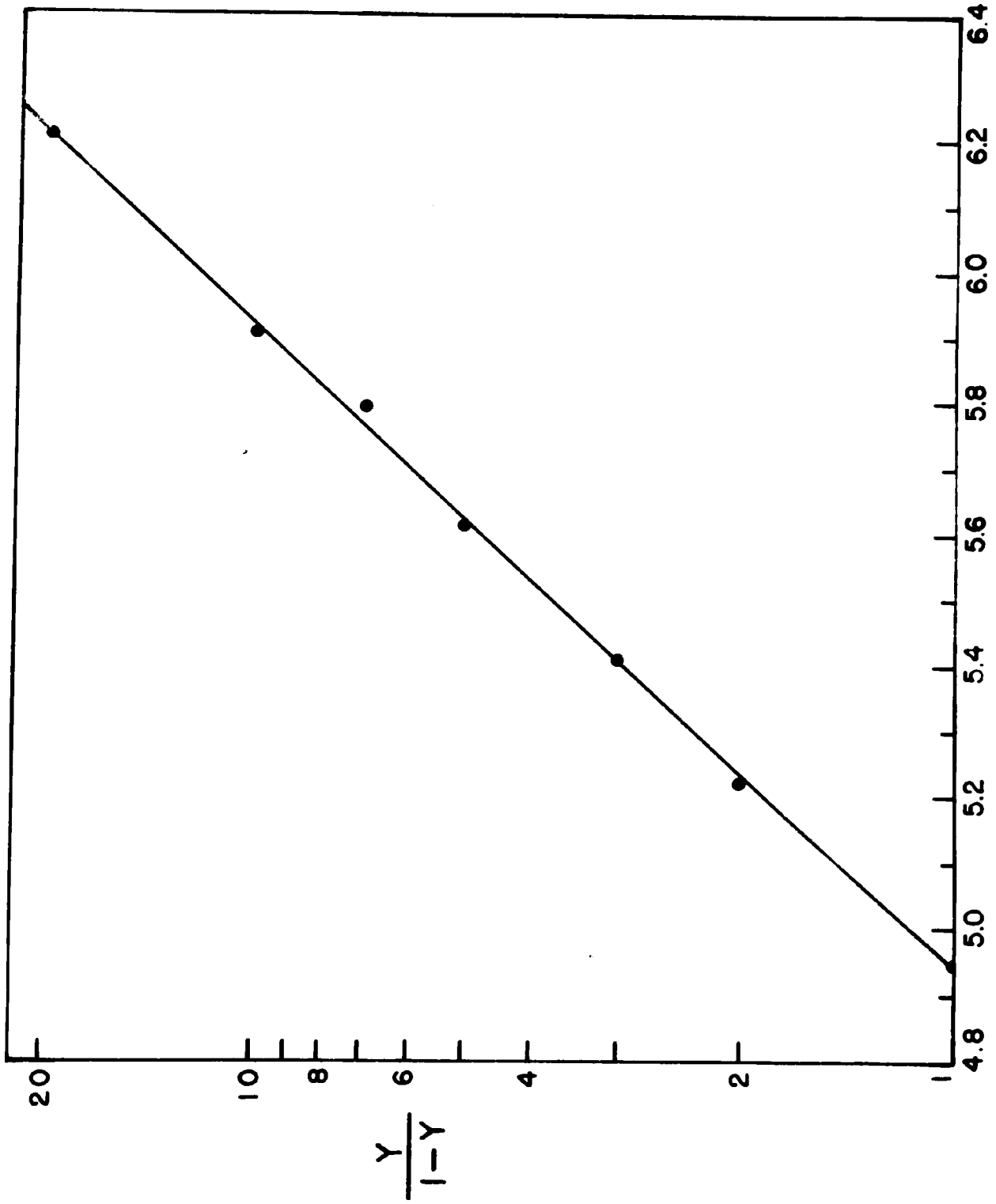


Figure 9

Figure 10

THE pH vs. ACTIVITY CURVE FOR TENSION

(20^{o} and 0^{o} C)

Abscissa: pH calculated via Henderson-Hasselbalch equation for every 4th experimental point of Figs. 5 and 11

Ordinate: per cent tension

Retractor penis: Left hand curve 20^{o} C, n = 1
Right hand curve 0^{o} C, n = 3

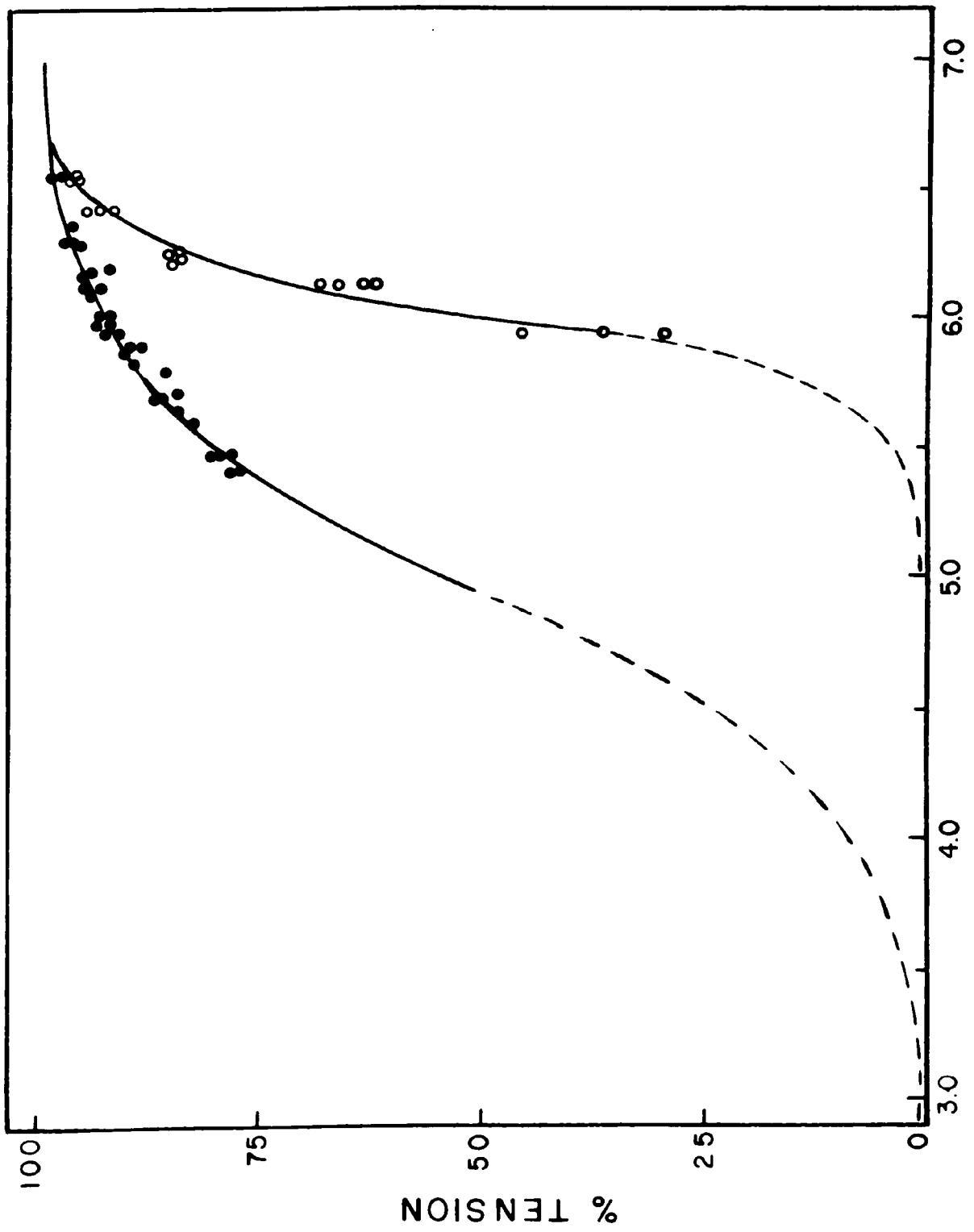


Figure 10

proper CO_2 concentration and pK_1' for this temperature were taken from the data of Harned et. al. in table I.

In addition to these experiments, which were performed with precautions to ensure strict reversibility, a number of experiments were performed in which the reversibility was not complete. These are the experiments with $NaHCO_3$ buffer reported in table 2 which, as pointed out, were done at the outset of this experimental study. It was from these data, as a matter of fact, that the need for relatively mild pH conditions became evident, if good quantitative data were to be obtained. In Fig. 11 these results are plotted as a pH vs. activity curve, with the same assumptions as before, i.e., complete equilibration of the external and internal bicarbonate concentrations and a "fixed intracellular base" of .76 mM. For the retractor penis muscle this practice does not need further justification, but for the frog sartorius the assumption of that amount of fixed base has no experimental substantiation. For the latter preparation, this entity could not be determined quantitatively and the reasonable fit of the data to the theoretical curve only suggests that this buffer fraction is not greatly different between the two muscles.

The results for the retractor penis muscles show a much greater variability than in the previous graph. It is also

Figure 11

THE pH VS. ACTIVITY CURVE FOR TENSION (EARLY EXPERIMENTS)

Abscissa: pH calculated via Henderson-Hasselbalch equation for experiments of table 1

Ordinate: per cent tension

● --- ● Retractor penis muscles
o --- o Frog sartorii

20^{o} C.

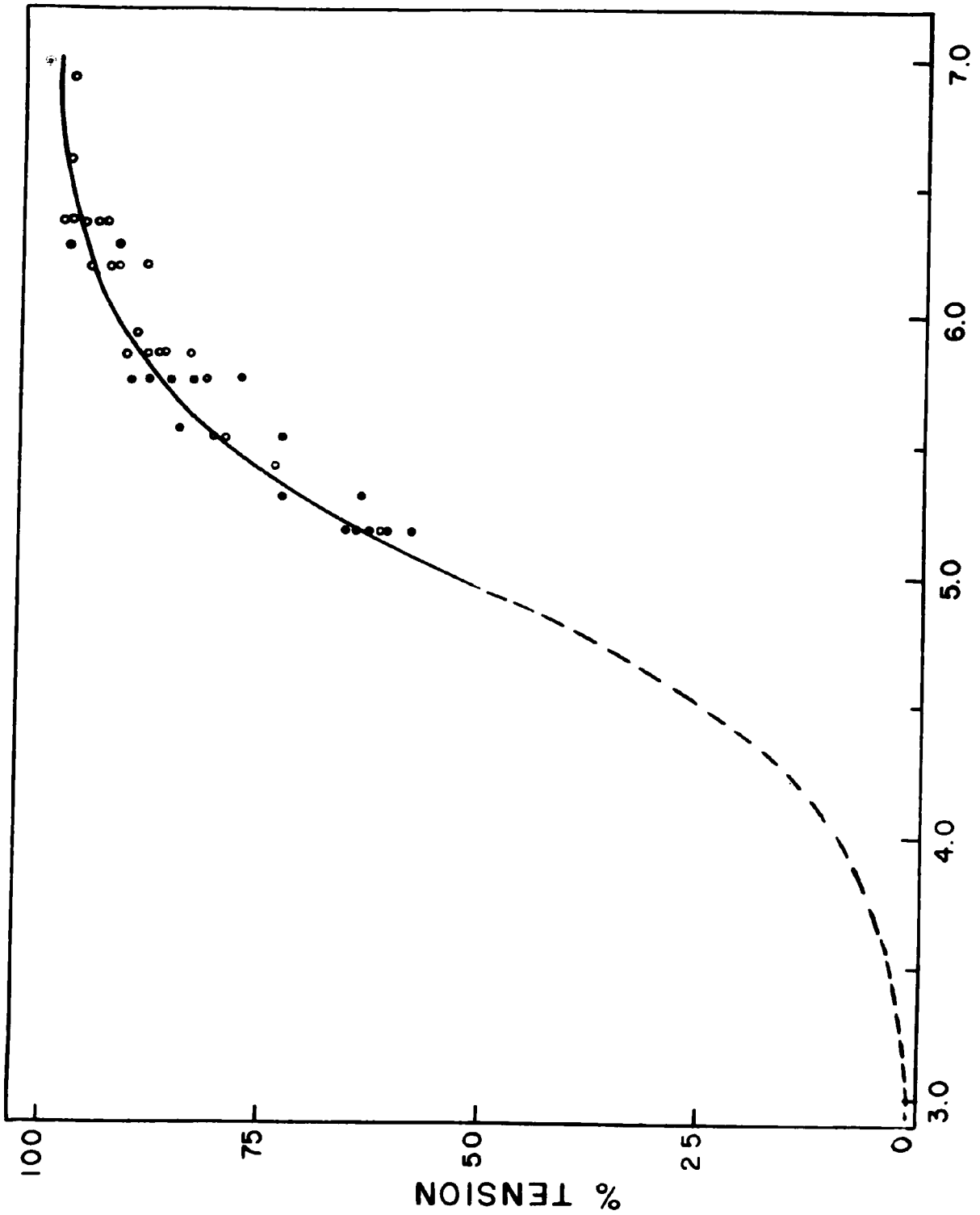


Figure 11

evident that the scatter does not fall symmetrically on both sodes of the curve; low values are more frequent than high ones. This must be attributed to the occurrence of an irreversible process to which a discrepancy between the control readings before and after exposure to the high CO_2 level also bears witness. The practice of using only the first control value for the calculation of this inhibition must in the presence of an irreversible process have given rise to this consistent deviation towards an increased amount of tension decrement. Nevertheless the correlation between the theoretical and the experimental values in Fig. 11 may be considered satisfactory.

The curve for the experiments at 20° C reaches 50% inhibition at pH 4.9. In analogy to accepted terminology this value can be considered as the pK of the contractile system at full activation. For the curve at 0° which showed a stoichiometric factor n = 3 the pK equals 5.9. Thus the fully activated and the activation-limited state do not only differ in respect to their values for n, but they also have widely different pK values.

This observation partially explains the fact that at 0° C it is possible to inhibit tension reversibly to a much greater extent than at 20° C. At the first temperature the intracellular pH has to be changed by only about one unit to produce an inhibition of 50%, while at 20° this change has to be

in the order of two units. These larger changes in the intracellular pH can easily cause irreversible damage either to the contractile system or to other components of the cell.

B. Excitability

The excitable system is most easily studied by the determination of the strength-duration curve. Not only does this relationship provide an easily established index to the excitable system contained in the cell membrane, but it is also an index which is closely linked to the functional activity of this membrane. The latter fact, therefore, attaches additional importance to any experimental results in that they may contribute to a better understanding of this distinct part of the muscle cell.

1. Intensity-duration relations

In Fig. 12 some typical S. D. C. data for the retractor penis muscle are shown for the two types of electrodes. In this presentation the logarithm of the duration of the stimulus in milliseconds is plotted on the abscissa against the minimum field strength or voltage needed for excitation in that time on the ordinate. The discontinuities indicate for each curve the point where the method of stimulating the muscle fibers changes. The part of the curve to the right represents direct excitation of the muscle membrane, while the portion to the

Figure 12

RUSHTON PLOT OF STRENGTH DURATION CURVES

COMPARISON OF M. L. F. AND STIGMATIC ELECTRODES

Abscissa: logarithm of the duration of the rectangular shock

Ordinate: logarithm of the voltage needed for effective stimulation

Retractor penis

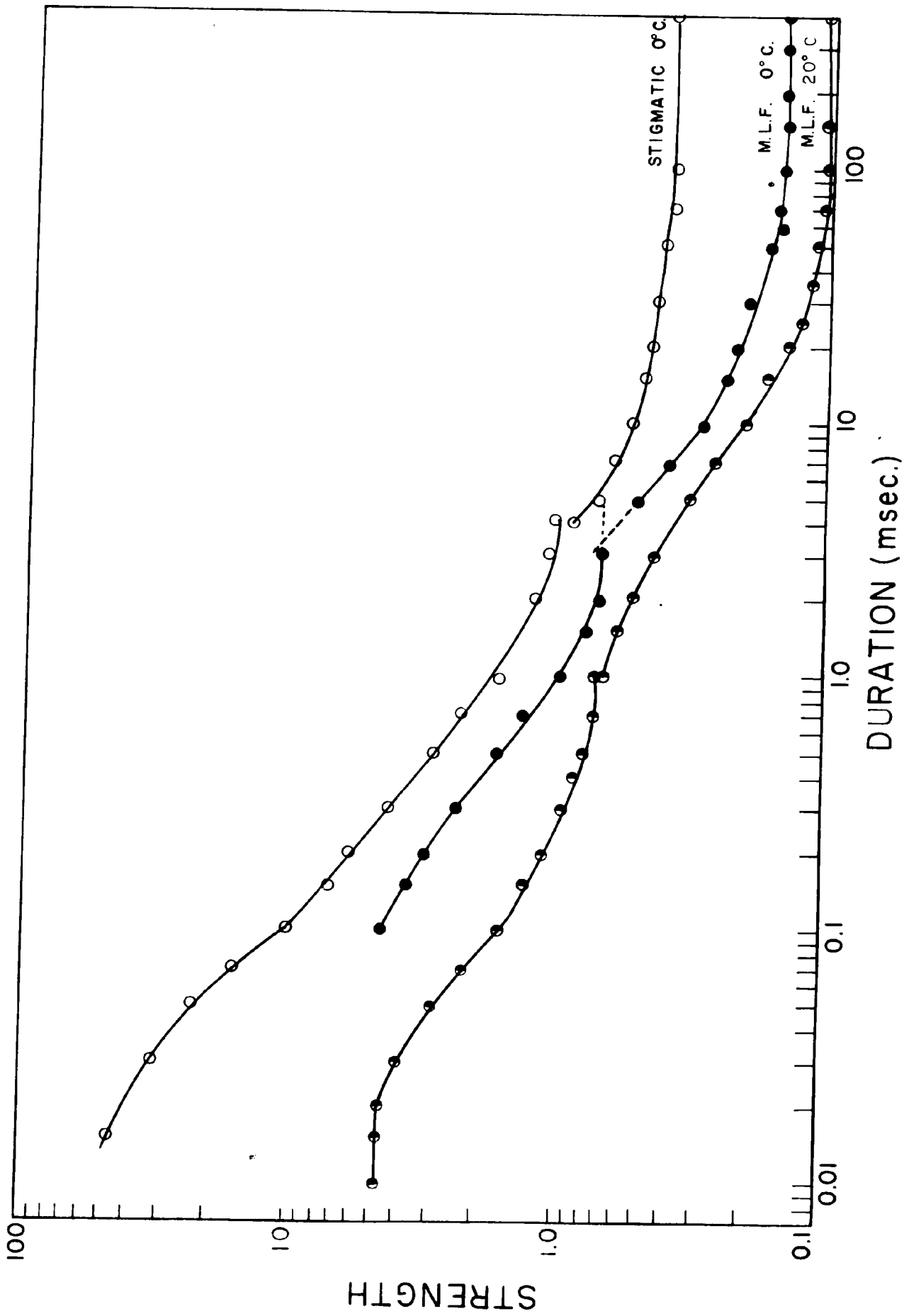

STIGMATIC 0°C.
M.L.F. 0°C.
M.L.F. 20° C
STRENGTH
100
10
1.0
0.1
DURATION (msec.)
0.01
0.1
1.0
10
100

Figure 12

left corresponds to the region of indirect stimulation of the muscle through intramuscular branches of its nerve supply. This interpretation of the complete S. D. C. of excised muscle originated with Lucas (1906) it was confirmed by many other investigators, while the point was finally settled by Rushton's convincing researches (Rushton, 1930, 1931, 1932a, b, c, d).

By blocking impulse transmission from the nerve to the muscle one can eliminate the nerve branch of the curve. The neuro-muscular junctions apparently are very resistant to blocking agents. The concentration required for complete block is three to four times higher than the level commonly employed for the frog sartorius or gastrocnemius (20-30 gamma/cc). Because these high levels tend to raise the rheobase, and also because the minimum curare concentration varies with some parameters such as calcium concentration, while, in addition, during the long duration of the experiment the drug might alter the excitability characteristics, curarization was not employed. Instead another method of graphical representation was used that promised to yield a useful parameter.

In Fig. 13 the same S. D. C.'s of Fig. 12 are plotted according to Blair's analysis. On the abscissa the duration in msec is plotted and on the ordinates the corresponding values of

Figure 13

BLAIR PLOT OF S. D. C.

COMPARISON OF M.I.F. AND STIGMATIC ELECTRODES

Abscissa: duration of the rectangular shock

Ordinate: log of the ratio of the voltage needed for stimulation to the difference between that voltage and the rheobase

Same data as Fig. 12.

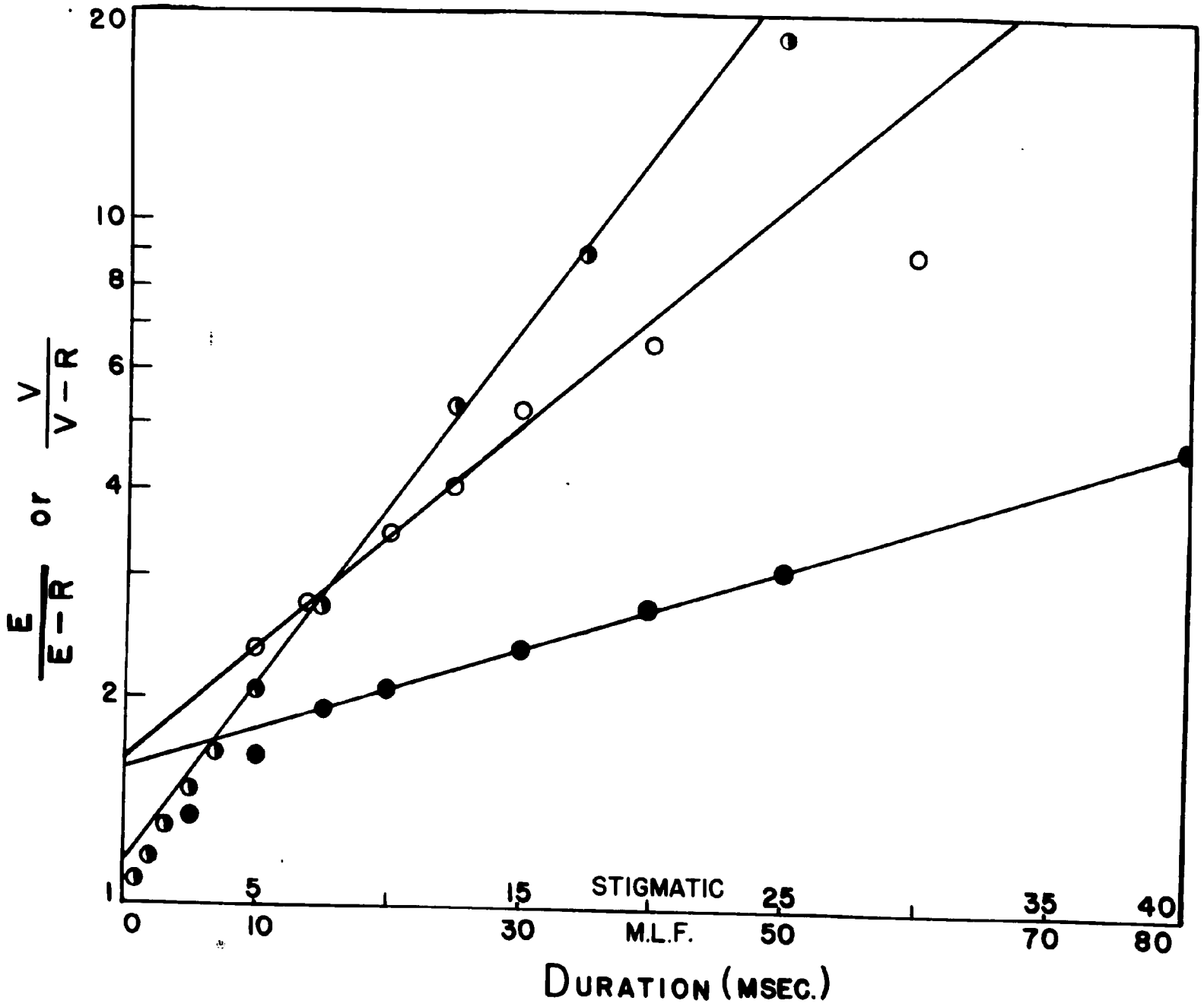

20
10
8
6
4
2
1
E/E−R or V/V−R
0
5
10
15
30
STIGMATIC
M.L.F.
25
50
35
70
40
80
DURATION (MSEC.)

Figure 13

(V/V-R) on a logarithmic scale. The relation is obviously linear for the larger values of t and a straight line is fitted to the points. The intercept of the line with the ordinate gives us the value of C while Blair's k calculated from the slope of the line is 27.2, 78.3 and 59.6 for M.L.F. and stigmatic electrodes at 0^{o} and M.I.F. at 20^{o} C respectively. The corresponding values of C are 1.60, 1.60 and 1.15.

As expected, the curves deviate considerably from straight lines at the longer and shorter durations but conform very adequately to the relation in the middle part. The values of C are obviously not equal to unity and the general fit of the straight lines is good enough to rule out a possible shift from unity due to experimental error. Subsequently a large number (76) of S.D.C.'s were determined under a great variety of conditions of Ca^{++}, K^{+} and H^{+} ion concentrations and of temperature. In all cases C was found to be larger than one and it can be decided that this constant truly differs from unity and that for the muscle membrane Blair's equation is more valid than Hill's.

Because of this last conclusion it was decided to report the results on the dynamic time constant in the form of Blair's k. It may be pointed out, however, that Hill's K is simply the reciprocal of that measurement. The difference in k as

determined with stigmatic and M. L. F. electrodes is in agreement with older observations (see Blair, 1941). The values for the stigmatic electrodes are always larger than for the M. I. F. stimulation, and increase with decreasing size of the stigmatic electrodes. From this point of view the results determined with the M. I. F. electrodes are more general and should be preferred as an index to the excitability.

In the presence of an increased CO_2 partial pressure the membrane is less easily excited. It is found that a shock of a certain voltage has to flow longer to produce a response or conversely that at a given duration of the shock an increased voltage is needed to make the stimulus effective. This means that the S. D. C. is displaced up and to the right.

2. CO_2 and excitability

In Fig. 14 a series of S. D. C.'s for a retractor penis muscle is plotted as log (E/E-R) vs. t. For this family of curves the bicarbonate concentration was 3.5 mM and the CO_2 percentage was varied. The experiments were performed employing the same procedure as the corresponding ones on tension variation. The reference solution was equilibrated with 1.0% CO_2 and the muscle was returned to it after each exposure to a higher CO_2 pressure. The S. D. C. was determined in each solution as well as in the reference solution before and

Figure 14

EFFECT OF CO_2 ON EXCITABILITY; BLAIR'S PLOT

Abscissa: duration of the rectangular shock

Ordinate: log of the ratio of the voltage needed for stimulation to the difference between that voltage and the rheobase

Results for one muscle with 3.5 mM bicarbonate with varying pCO_2 as marked in the margin. Letters indicate chronological order of the determinations. Retractor penis 20° C.

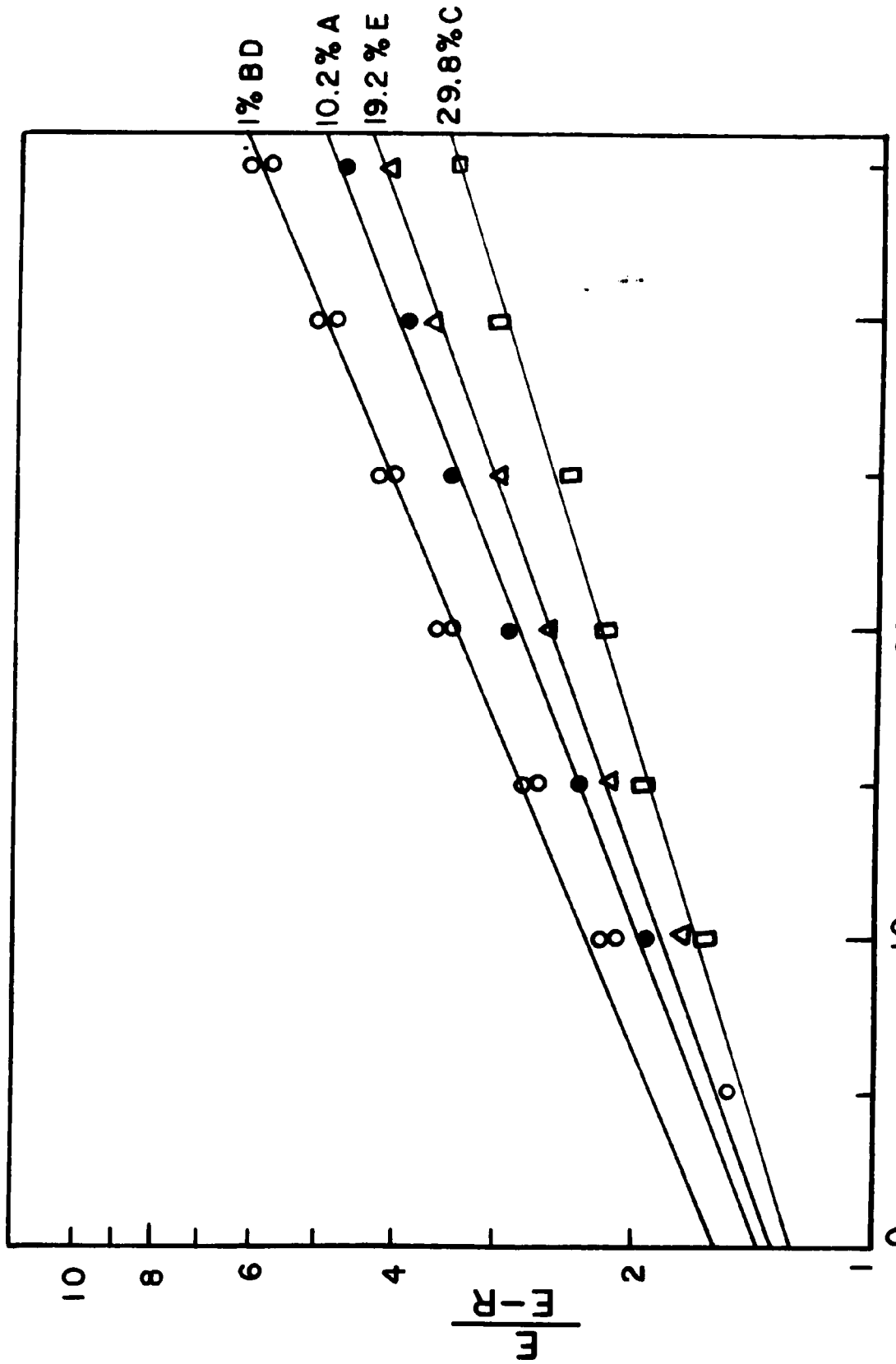


Figure 14

afterwards. For these curves the time constant k was calculated and the results for different CO_2 pressures expressed in percentages of the value in the reference solution in parallel to the experiments on tension development. The results of these calculations, plus ones on the same muscle for 0.5, 1.5, and 5.0 mM $NaHCO_3^-$, are shown as a Fisher-Ohnell plot in Fig. 15, where y = fractional value of k remaining and (1-y) = fractional value of k inactive. The straight lines were fitted through the points and again have a slope of n = 1, and are described by the relation $y/1-y = n [CO_2]$.

The results on the excitability turned out to differ from those on the tension in the reproducibility between preparations. This is illustrated in Fig. 16 where the results of 3 muscles (A the same as Fig. 15) are shown all at one bicarbonate concentration (3.5 mM). It is clear that the slope of the lines is quite reproducible, in other words, that the stoichiometric factor n is the same for all preparations. Agreement among the data for each muscle is satisfactory. The position of the lines is, however, quite different from one preparation to the next. In view of the good agreement of the data for each muscle, these differences are likely to be real and not due to experimental error. The difference lies apparently in the value of the equilibrium constant for the combination of the inhibitor with the enzyme.

Figure 15

FISHER-OHNELL PLOT OF THE CO_2 EFFECT ON BLAIR's k

Abscissa: log of the pCO_2

Ordinate: log of the ratio of the uninhibited to the inhibited fraction

From left to right: 1.5, 2.5, 3.5 and 5.0 mM $NaHCO_3$

Retractor penis, 20° C

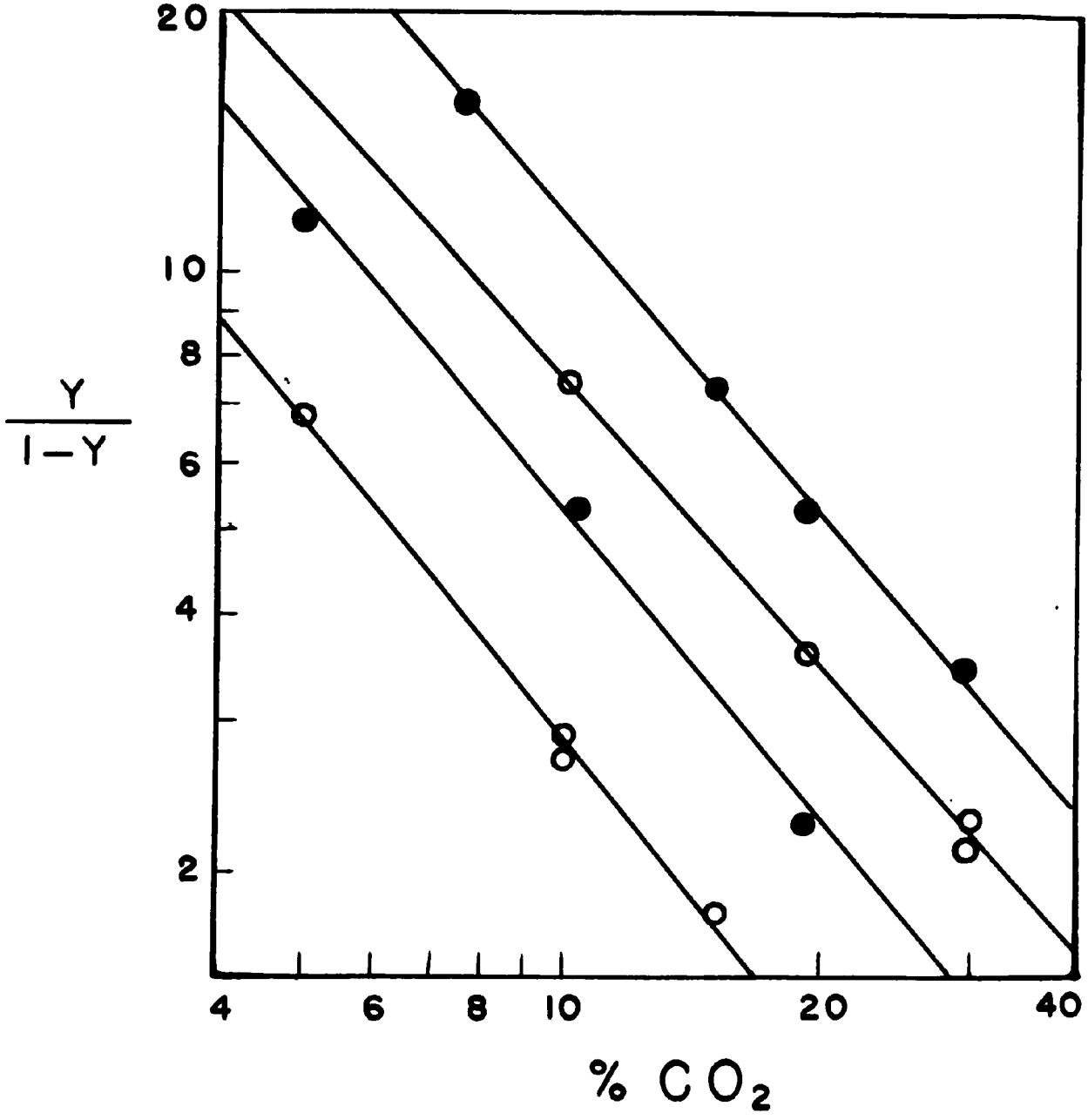


Figure 15

Figure 16

VARIABILITY OF THE CO_2 EFFECT ON BLAIR'S k

Abscissa: log of the pCO_2

Ordinate: log of the ratio of the uninhibited to the inhibited fraction

Three retractor penis muscles all with 3.5 mM $NaHCO_3$ 20° C.

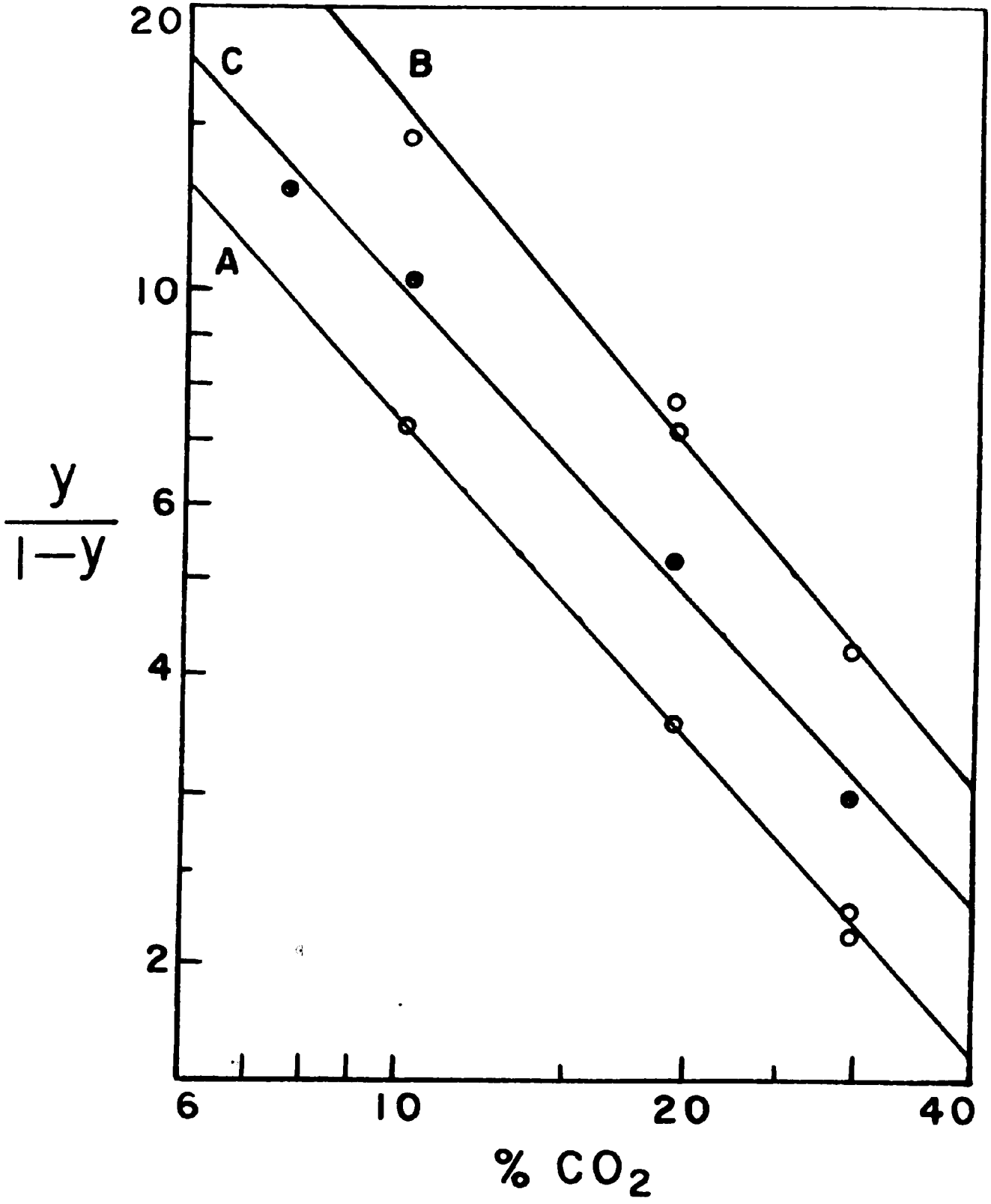


Figure 16

In Fig. 17 the relation between $NaHCO_3$ and pCO_2 at a constant (y/1-y) value is plotted for four muscles. For muscle A this amounts to reading horizontally across the graph of Fig. 15 and noting the pCO_2 for each bicarbonate concentration. The curves marked B and C are for the same muscles marked B and C in Fig. 16, but each also at other bicarbonate concentrations (i.e., B at 0.5 and 1.0 and C at 1.5 mM). Curves D and E are for a fourth and fifth muscle not previously discussed.

The intercept of the extrapolated parts of the five experiments are at 0.25, 0.07, 0.08, -0.07 and -0.11 mM $NaHCO_3$, for an average of -0.04 mM. This shows that the time constant of excitation changes in direct relation to variations in the bicarbonate content of the external solution. In other words, no extra intra-cellular base should be added to the external concentration to describe the dependence of Blair's k on pCO_2.

From the data on any of these muscles it is possible to construct a pH vs. activity graph in analogy to those calculated for the data on tension development. The same procedure is followed. First the relation between CO_2 and external bicarbonate for several equal levels of inhibition i.e., equal values of (y/1-y) is found by reading horizontally across the graph of Fig. 15 and noting the pCO_2 needed at each HCO_3^- concentra-

Figure 17

THE pCO_2 NEEDED FOR EQUAL INHIBITION OF BLAIR's k AT VARIOUS BICARBONATE CONCENTRATIONS

Abscissa: mM $NaHCO_3$ in the Ringer's

Ordinate: pCO_2 necessary to give equal inhibition, i.e., constant (y/1-y) value of Fig. 16. (y/1-y = 6).

Retractor penis, 20° C

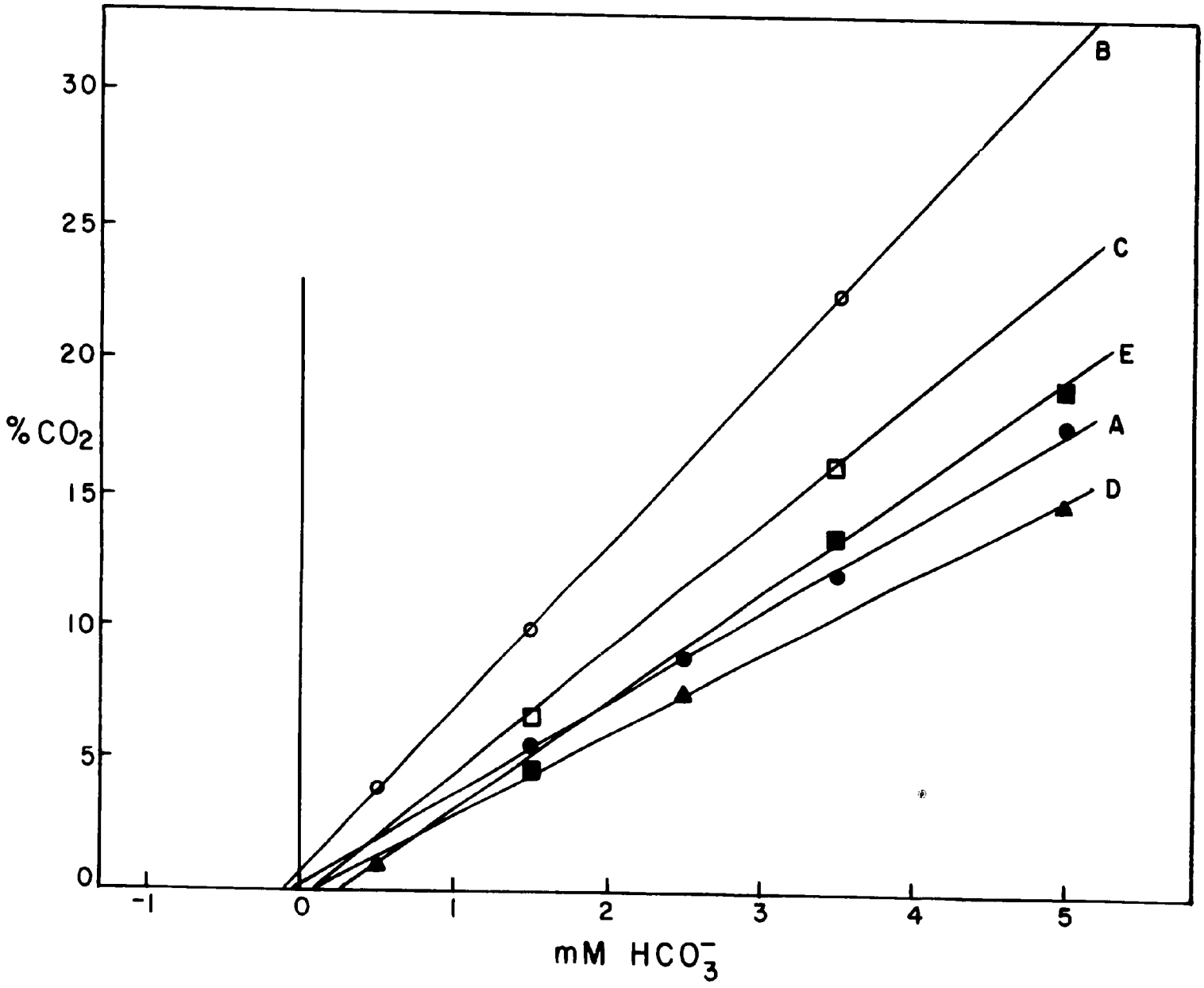


Figure 17

tion. (This was done already for $y/1-y = 6$ for the five muscles described so far.) For the contractile system the intercepts of the resulting lines with the ordinate at zero external base were then determined and the pH calculated from those figures employing .76 mM HCO_3^- as the base. In the present situation this procedure has no value because the lines intersect the abscissa at or close to zero bicarbonate. Any arbitrary concentration of HCO_3^- can be used, however, with equal validity. Thus, 4.0 mM bicarbonate was chosen, an ordinate was erected and the pCO_2 values of the intercepts of it with the lines for equal $(y/1-y)$ values were read off. From these pCO_2 values and using 4.0 mM as the base concentration, the pH at each level of inhibition was calculated. The results are plotted in Fig. 18, as pH vs. Blair's k, the latter expressed as per cent of the k value at the control pH. The letters refer to the 5 muscles analyzed in Figs. 16 and 17. Only the points for Curve A were connected by a curve, which is shown as a broken line in the extrapolation to lower pH values. To reduce the number of points on the graph curves C, D, and E are only represented by two points, while for the most extreme one, B, a larger number of points is displayed. The dotted curves are the ones of Fig. 10 for the tension and are added for comparison.

All the muscles show the same type of relation between pH and Blair's constant, which is not surprising as the stoichio-

Figure 18

THE pH VS. ACTIVITY CURVE FOR BLAIR'S k

Abscissa: pH calculated from the Henderson-Hasselbalch equation

Ordinate: per cent of k value at reference pH

Retractor penis 20° C

Solid line for muscle A, points labeled B, C, E, D refer to the 5 muscles in Fig. 17. The dotted curves are for n = 1 and n = 3 inhibition of tension.

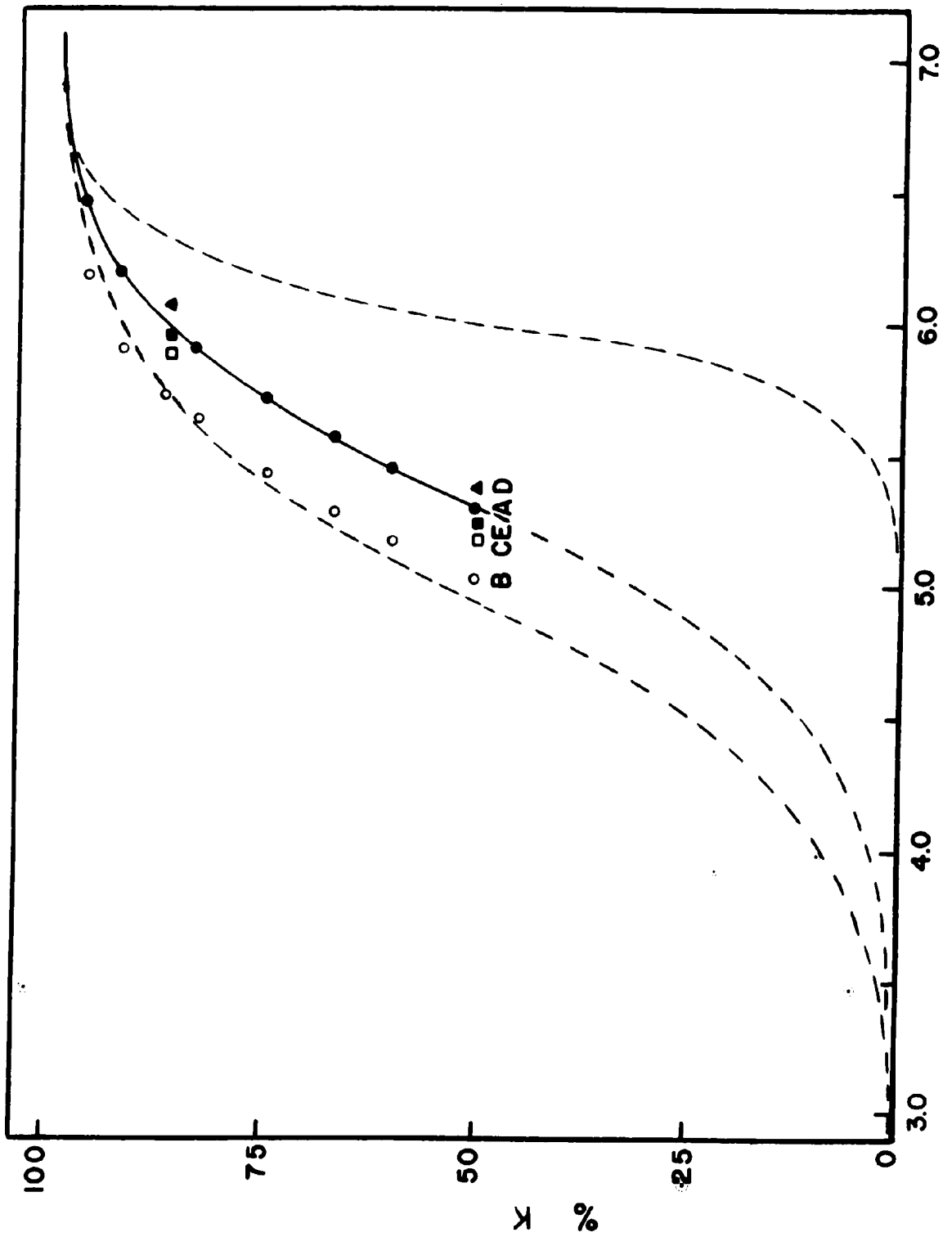


Figure 18

metric factor n was found to be close to one for all. It is interesting to note that the variability between muscles which appeared to be quite large in Figs. 16 and 17 actually amounts to a range of pK values of only 0.35 pH units. It is also interesting and perhaps significant that the pK's for tension and for Blair's k fall close together.

It may be concluded that, although the pK values for tension and Blair's k fall close together and that at 20° C both these parameters are inhibited by H^+ ions with a stoichiometric factor equal to one, the systems studied are quite different; the latter varies directly with the external bicarbonate while the former shows the participation of an extra "intracellular base" fraction. It can also be concluded that the inhibition of tension is not a secondary phenomenon produced by failure of, or changes in the excitability of the membrane.

CHAPTER V

DISCUSSION

The present study is aimed in the first instance at establishing, for the specific case of striated muscle, the answer to the question whether the physiological action of CO_2 on tension and excitability is mediated through its effect on the internal pH of the cell. From the results of Smith on cardiac muscle this mode of action was anticipated in regard to contraction. For the excitability it is known that at low values of external pH the membrane may fail to respond when the muscle can still be made to contract by massive stimulation (Hill 1955, Goodall, 1956). This evidence suggested the possibility of a dependence of the membrane excitability on the external, rather than the intracellular pH.

From the beginning of the investigation it was hoped that it would prove to be possible to establish for striated muscle the quantitative relation begween intracellular pH and muscular contraction. Such a study would be of great interest for the sake of correlating the in vivo pH effects with those produced in various isolated parts of the contractile system, such as the glycerol-extracted fiber.

The two measurements chosen for the study of the effects of CO_2 on muscle were the tetanic tension and the dynamic time constant for excitation, k, as defined by Blair (1941).

Tension is a characteristic property of the myofibril within the cell, while the time constant k relates to the depolarization of the cell membrane, which process is generally believed to occur in the intracellular-extracellular interface. By choosing these structurally distinct functions for study, it was hoped that the results would throw light on the validity of calculations of the internal pH. At the same time and regardless of this hope, it was evident that such studies would yield valuable information on the concomitant behavior of these two important functional aspects of muscle.

A. The Mode of Action of CO_2

As pointed out before, the ways in which different partial pressures of CO_2 can affect the maximal tension produced by the muscle are three: (1) by a direct effect of either intra- or extracellular H_2CO_3 or CO_2 in physical solution, (2) by an increase of the extracellular H^+ - ion concentration or (3) by an increase of the intracellular H^+ - ion concentration. The two agents named in the first possibility are both directly dependent upon the CO_2 partial pressure. The concentration of both of these is in no way dependent upon the bicarbonate concentration at constant ionic strength, as was shown by the work of Harned and Davis. If the tension or Blair's k were diminished by the concentration of either CO_2 or H_2CO_3, the fall in tension should be proportional to the CO_2 partial pressure.

From Table 2 and more extensively from Figs. 2 and 3 it is apparent that no such relation exists: the same CO_2 - O_2 mixture can have widely divergent effects.

An increase in the CO_2 partial pressure will also produce an increase in the H^+ - ion concentration by reactions (2) and (3) above. The possibility that the decreased external pH diminishes the tension output of the muscle was excluded by the experiments reported in Table 2. Examination of that table and especially comparison of line 2 with line 3, 4 with 5 and 11 with 12 shows that approximately equal changes in pH have greatly different effects when brought about by changes in the phosphate buffer or by increases in the pCO_2. These observations rule out the possibility of the external pH affecting the contractility directly, leaving as the only remaining choice of a direct agent the increased intracellular H - ion concentration. For the excitability the case is different.

B. Quantitative Relations Between pCO_2 and Excitability

The analysis of the depression of the excitability was performed by the study of the effect of higher CO_2 pressures on the dynamic time constant of the membrane, k, as defined by Blair (1932, 1941). Preliminary studies indicated that this entity as well as the membrane constant C could be determined with relative ease without the use of neuro-muscular blocking agents and were, therefore, well suited to this analysis. In

the course of this preliminary work, it was observed that for the retractor penis muscle stimulated by M. L. F. electrodes, Blair's mathematical expression of the strength-duration curve was more valid than Hill's. This became evident from the fact that the constant C, which Hill set equal to unity, was found to be always larger than one, under a wide variety of temperature and ionic conditions. In the subsequent study of the effect of CO_2 no specific pattern of variationof C could be established, while k was found to change systematically with the CO_2 partial pressure.

The investigation of the effect of carbon dioxide on k brought two differences from the behavior of the tetanic tension to light. In the first place, the diminution of k with increases in the CO_2 partial pressure was found to proceed without the presence of a fixed intracellular base. In the second place a great variability in sensitivity was found among individual muscles, which cannot be ascribed to experimental error. The latter feature is reminiscent of the findings of Smith for isotonically shortening turtle cardiac muscle and is in sharp contrast with the inhibition of tetanic tension which showed such good reproducibility that the data from several muscles could be pooled for quantitative analysis. No explanation of this finding can now be given. At the most it can be suggested that the source of the variability may lie in subtle differences between

the hormonal and nutritional status of the turtles.

The difference in the response of the excitability to CO_2 in relation to the external bicarbonate level and that of the tetanic tension is striking indeed. While for the latter function the occurrence of a fixed intracellular buffer equal to 0.76 mM bicarbonate was established, no such buffer fraction was found for the excitable system. For this system it was found that the target of the action of the H^+ - ion was affected by CO_2 changes in a manner directly dependent on the external bicarbonate concentration (see Fig. 17).

These two differences in response to CO_2, one qualitative, the other quantitative, between the excitable system and the tension-producing mechanism, are such that the possibility that the effects observed in the latter are reflections of changes in the former can be excluded.

C. Quantitative Relations Between pCO_2 and Tension

The analysis of the quantitative data on the CO_2 induced decrements of tension led us to three conclusions: (1) that the extent of the effect of CO_2 is dependent on the bicarbonate concentration with which the muscle had previously been equilibrated, (2) that the relation between external buffer and the tension decrement produced by an increased pCO_2 was such that it could only be explained by assuming that the externally applied bicarbonate penetrates the muscle cell and there

exerts a buffering action together with a certain amount of fixed intracellular buffer, and (3) that the action of CO_2 on tension is mediated through changes in the intracellular pH.

The first conclusion, i.e., that the extent of the effect of CO_2 is dependent on the external bicarbonate concentration with which the muscle has been equilibrated, was interpreted as meaning that bicarbonate penetrated the muscle cell. This is in agreement with the findings of Brookens (1933), Fenn and Cobb (1934) and Fenn and Maurer (1935) who studied this question by direct chemical analysis (see Chapter II). These studies showed that the intracellular bicarbonate concentration of excised, frog muscle does indeed vary with the external one and the best evidence indicated that the ratio of the external to the internal level is maintained as 1.2 to 1.0. The present result is therefore in keeping with these previous findings.

The amount of the fixed intracellular buffer, mentioned in conclusion 3 above, was found to be equal to the amount of bicarbonate that would penetrate the muscle from a Ringer's solution with 0.76 mM bicarbonate. From the work of Fenn and coworkers cited above, it seems likely this amount is not equal to 0.76 mM, but is probably a fraction of it very close to unity. The actual, chemical make-up of this buffer fraction is, at this moment, completely undisclosed.

In these terms it is, however, of more than academic interest. Smith (1925) determined this fixed base in marine eggs for several acids (carbonic, acetic and propionic), and found that the size of the fixed base term varied. Recalculated, however, on the basis of the varying buffering action of these acids he showed that these terms were all identical (0.9 mM) when expressed in terms of bicarbonate. This molarity was the same as the value found with the bicarbonate - CO_2 system itself.

For the isotonically shortening turtle auricle, Smith (1926) found a fixed intracellular base corresponding to 2.2 - 2.8 mM bicarbonate. He also noticed that the slope of the change of (ΔH CC /Δ % CO) with bicarbonate varied greatly between preparations the least sensitive being four times less responsive than the most sensitive one. These results are quite different from the findings reported here. It may be argued either that cardiac muscle behaves quite differently from skeletal in its response to intracellular H^+ -ion changes or that in the heart muscle another process is primarily affected.

The rather surprising results of the linear relation between CO_2 concentration and tension or shortening at room temperature, as revealed by Smith's analysis, are probably partly due to the fact that the log y/1-y is related to the pCO_2 with a slope equal to 1 (see Fig. 3) and partly to the fact that the

CO_2 concentration was varied over a limited range only. In contrast with inhibition mechanisms which show a stoichiometric factor of a higher power (2 or 3, for instance), Smith's analysis of a system for which n is unity shows an almost linear relation at low inhibitor concentration which, however, becomes curvilinear when the level is increased.

The fixed intracellular buffer should, from a functional point of view, be directly comparable to the buffer capacity of the cell. But while 0.76 mM HCO_3^- produces buffering power, β, of only 0.00013, the generally accepted values for muscle tissue are probably at least 100 times larger, even when differences in experimental technique are taken into consideration (see Chapter II, section B). This discrepancy is far beyond any that can be ascribed to experimental error.

In the introduction an attempt was made to either reconcile the difference between Smith's value of 2.4 mM and the accepted figure, or to find at least some explanation for it. Although those considerations tended to diminish the discrepancy between the two determinations of the buffering capacity, an actual difference was thought to be present. The data reported above tend to confirm the existence of a true difference between the two sets of values.

All the aforementioned considerations of the intracellular pH have treated the cell as a homogeneous, if complex,

solution. The actual conditions are, however, a far cry from such a simple concept. The striated muscle cell has an even more complex inner structure than most other cells, and this structure is essential to the performance of its contractile function. The concept of an orderly arrangement of actin and myosin within the myofibril, as brought to light by the interference microscopy technique of Huxley and Hanson (1957 a, b), emphasizes the importance of the relation of the subcellular, "molecular anatomy" to function. In such a structure the local charges on the contractile proteins, and the ions adsorbed to them as well as those contained in the ionic clouds, can easily be imagined to modify greatly the H^+ - ion concentration of the myofibril from the rest of the cell and even to produce differences within different regions of the myofibril itself. In relation to this, it is also interesting to consider the fact that at pH 7 a volume of $1 \mu^3$ contains only 60 H - ions and a volume equal to that of the myofibril, within one period of striation, only about 15 to 20. From the data on the molecular weights and sizes of myosin and actin, as compiled by A. G. Szent Gyorgyi (1955) and using a figure of 75% for the water in the myofibril, we can very roughly estimate the total number of actin and myosin molecules as 250 for that same volume of the myofibril. It is clear that at these dimensions a certain pH value has more the meaning of a time average

than a space average.

The discrepancy between the overall acid-base relations of the cell and that part of it whose changes are expressed in the functioning of the contractile elements does, therefore, not necessarily constitute a contradiction, but should rather be considered as the result of the inhomogeneity of the cell. Notwithstanding this conclusion, it is worthwhile to discuss tension as a function of pH.

Before doing this, two possibilities of indirect effects of high CO_2 remain to be considered. There is first the possibility of changes in the intracellular potassium concentration with changes in the pH. Because of its importance to theories of membrane equilibria of the Gibbs - Donnan type, such changes were extensively investigated by Mond and Netter (1930, 1932), Netter (1934) and Fenn and Cobb (1934, 1935). These studies show a small shift of K^+ - ions from the muscle to the Ringer's over a period of hours in response to an increased pCO_2. For example, a loss of 4.8% occurs in the frog sartorius in response to a change of pCO_2 from 1.0 to 9.8%. These changes take place, however, on a time scale completely different from the effects studied here, being maximal 5 to 10 hours after the increase of the acidity, and they can therefore be eliminated as the cause of the CO_2 effect of muscle tension. The other effect of an increased

pCO_2 is the breakdown of creatine phosphate, a process first observed by Gerard and Tupikov (1931) in excised nerves. These authors were led to their study by the work of Meyerhof and Lohmann (1928) who observed this hydrolysis in acid solutions in vitro. In subsequent years this phenomenon held an important place in considerations of muscle physiology because of the central role ascribed to creatine phosphate. The papers by Root (1933) and Meyerhof, Mühle and Schultz (1932) which may be considered the final word in this issue, show that the increased CO_2 produces a hydrolysis of creatine phosphate which is complete in 4 to 5 hours. From the length of this time span, as well as from the fact that decreases in phosphocreatine are not at first accompanied by a decreased tension output, it is clear that the rapid decrements in tension observed in this study are not related to the splitting of phosphocreatine as a consequence of the acidification of the intracellular milieu.

In the absence of other relevant effects of CO_2 on muscle, it can be stated that our conclusion, that the effects on tension development are produced by the increased, intracellular H^+ - ion concentration acting directly upon the contractile mechanism, is correct.

D. The pH Dependence of Tension in Muscle and in Glycerinated Fibers

The dependence of tetanic tension on intracellular pH was

depicted in Figs. 10 and 11. In those graphs the exact position of the curves on the pH axis is not certain. The calculations were based upon complete equilibration of the external and internal HCO_3 concentration plus an additional 0.76 mM of bicarbonate. This practice may have resulted in a slight error in the pH values, as it was found by Ferm and coworkers that excised frog muscles do not come to absolute equilibrium with external bicarbonate. The ratio of external to intracellular HCO_3^- was found to be as 1.2 to 1. Such a discrepancy would, however, not influence the slope of this curve but only its position on the pH scale. In addition to this it is unknown at what pH value the buffer system which provides the extra 0.76 mM buffering is set in the intact muscle. The exact value of the pK for either the n = 3 or the n = 1 relation can, therefore, not be determined.

However, the differences between the two pK values and also between the slopes of the two curves are significant. Both these features point up the fact that the relation is quite different under these two conditions while the internal consistancy of the data excludes the possibility that this difference is due to experimental error. These two features provide, therefore, a satisfactory basis for comparison with the data on the glycerinated fibers.

The study of glycerinated muscle fibers has produced a large body of information which suggests that this preparation

closely resembles the contractile machinery of the muscle stripped from its excitation system and lacking the capacity for metabolic synthesis. Various evidence indicates that these fibers, produced by the prolonged treatment of small, excised muscle bundles with 50% glycerin in water at temperatures below 0° C, contain little else besides the cyto-architecture of the intact cell. Retained within these fibers are the large structural proteins, such as actin and myosin, various transphosphorylases and other enzymes, while the readily diffusible substances of smaller molecular weight have been leached out. The fibers can be made to contract by the addition of a suitable, high-energy phosphate donor such as ATP and in some instances be relaxed again by creatine phosphate (CP), "Marsh factor," phosphoenol pyruvate and others. All of the contractile behavior resembles very closely the contraction of intact muscles and in this sense deserves comparison with data of this investigation.

Recently the work of Goodall and A. G. Szent Gyorgyi (1953) first established the importance of pH as a factor in the behavior of glycerinated fibers in solutions containing both ATP and CP. They found that relatively freshly prepared fibers would relax in the presence of CP if the pH was dropped from 7.5 to 6.0 The preparation apparently depended on the presence of an enzyme which was either only slowly washed out or was destroyed upon continued standing in the glycerine-

water mixture.

This work was greatly expanded by the investigations of Brown and Goodall (1956) and Brown (1957). In the fibers prepared by Goodall, which he terms "A-fibers" it was found that addition of small amounts of histidine buffer (5 mM, pH 6.8) and sodium cyanide (2 mM) was adequate for the preservation of the relaxing factor's activity for several weeks instead of a few days. This speaks in favor of the view that the loss of this activity is due more to the denaturation of an enzyme than to its diffusion out of the fiber.

The effect of pH on tension was studied in these "A-fibers." The Fisher - Ohnell plot indicated a stoichiometric factor $n = 3$ for the effect of H^+ - ion upon the tension when ATP alone was present. The relation between tension and pH is the customary s-shaped curve and it is, therefore, possible to speak of a pK for the system at half tension, which in this case is equal to 6.15. The presence of CP shifts the curve to the right in accordance with the expression

$$pK = 6.45 - 2 \log \frac{CP}{10}$$

where CP is expressed in millimoles per liter. Decreases in the ATP concentration shift the curve to the left in accordance to a similar expression relative to ATP. Otherwise the curve remains quantitatively identical, or in other words, n is still equal to 3. The addition of AMP to the system shifts the curve

to the left, again leaving n constant. From these results it is obvious that the exact position of this curve on the pH axis is highly dependent on the levels of AMP, ADP, ATP and CP while the stoichiometric factor remains constant.

Brown and Goodall (1956, see also Brown, 1957) concluded that the effect of the pH was upon the activation of the contractile system, as had been suggested also by Goodall and A. G. Szent Gyorgyi from measurements of the elastic modulus and from temperature studies. This conclusion makes it, therefore, necessary to view the activation of the contractile system as involving three individual sites on the contractile protein, each of which can be blocked by combination with an H^+ - ion.

Brown has also shown that in fibers where a relaxing agent such as phospho-creatine is not effective, the tension falls with increasing acidity with a stoichiometric factor equal to 1. These fibers, which are the more usual ones, show no sign of deactivation during acidification and the pH effect is considered to be directly on the capacity of the completely activated actomyosin to develop tension. In other words, when activation is complete, the inhibition of tension by H^+ - ions proceeds on a 1 to 1 basis.

In comparing the glycerinated fibers with intact muscle, the significant aspects are the pK values and the slopes. The pK of the fibers with a slope n = 1 in respect to H^+ - ion inhibition is very stable and falls at approximately pH 4.8 - 5.0.

In the fibers with n = 3, the pK value is dependent on the concentration of ATP, CP, AMP and ADP. In the intact retractor penis muscle the concentration of both ATP and CP is approximately 5 mM, while the AMP and ADP levels are negligible (Davies, 1957). At such concentrations the pK value of the n = 3 fibers falls in the region of pH 6.0 (-0.2). The two pK's are, therefore, approximately 1 pH unit apart. This is also the amount of separation for the n = 3 and n = 1 systems found in the present studies, where the difference is 5.90 - 4.95 = .95 units. In the live muscle the pK value for the n = 3 relation is also the more alkaline one.

Of the two characteristic entities, the pK and the stoichiometric factor n, the more significant one is the latter. In "A-fbiers" where n = 3 it is shown that at pH 7 the variation of tension with the temperature follows a curvilinear relation resembling that of the intact muslce (Fig. 1). In the glycerinated fiber this variation of tension with the temperature yields from the Arrhenius plot a μ value of 36,000 cal. The data on the temperature dependence of tension in the intact muscle has a similar value of μ as calculated from that plot. In the glycerinated fiber, this value clearly relates to the activation of the contractile actomyosin. In the retractor penis muscle, where a similar μ of 36,000 cal. exists, it seems appropriate to assume that this is similarly controlled by the

development of activation. In other words, the increase of tension with temperature up to the plateau level is controlled by the activation process. There is evidence in the pH relation of tension in this study which bears on this subject. If our views are correct, there is the possibility that at 0° C, where from the above it would appear that we were dealing with partial activation, i.e., 50% in summer turtles, we would be dealing with the effect of pH on activation. It is of interest, that under those conditions the relation between tension and pH is characterized by a slope $n = 3$. The accuracy of the present data is unquestionably adequate to establish this stoichiometric factor between tension and H^{+} - ion concentration. It seems evident, therefore, that at the low temperature, where the activation of inactive actomyosin appears to be controlling tension, both the data from the intact muscle and the glycerinated fiber are in agreement.

When these studies were undertaken it was the intention, as indicated before, to attempt to obtain data on intact muscle which would be sufficiently quantitative to permit a physico-chemical analysis in terms of the intracellular pH. The purpose of this was to present some feature of an intracellular reaction within the parameters, which are usually used in handling isolated enzymes, proteins or extracted organoids of one type or another, and it appeared that an essential function could be performed by attempting to relate some physico-

chemical properties of them to the intact system. The results of this study have permitted close comparison with those on the glycerinated fiber, pointing out similarities between the two, while they would also indicate that the latter preparation is more than a "model" of the contractile system.

It is clear that much more evidence must be accumulated on the living muscle under conditions where the development of the active state appears to control total tension, i.e., in the lower temperature range. The part of this study concerned with the pH effects on tension at 20° C, where one is dealing with the completely active state, is also of great interest. In the retractor penis muscle the plateau tension is sustained from 17° to 38° in summer animals. Although the bulk of the data in this paper deal with the effects of pH on the plateau tension at a temperature 3 to 10° above the attainment of that plateau, it should be valuable to investigate the same relation at a higher temperature. Furthermore, there are muscles, such as the catch muscles of the pecten, which are well known to sustain tension for long periods with very low energy exchange, a fact that has given rise to speculation about a "lock" or "catch" mechanism. As a point of departure for future research, it would seem most important to examine the relation of tension to pH in this muscle. From the above studies it seems probable that it is operating well beyond the control of the activation system.

CHAPTER VI

CONCLUSIONS

1. Increasing CO_2 partial pressures produce a drop in excitability and in contractility in striated muscle.

2. The effect on tetanic tension is mediated through changes in the intracellular pH.

3. The change of the tension output with increasing CO_2 partial pressures, in muscles equilibrated with different bicarbonate concentrations, proves that the intracellular pH varies in accordance with the view that the bicarbonate penetrates the muscle fiber and there acts as a buffering base in addition to an intrinsic, "fixed, intracellular" base.

4. The "fixed intracellular" base equals in buffering capacity the amount of HCO_3^- which would penetrate into the cell during equilibration with 0.76 mM bicarbonate.

5. The results of this investigation, as well as the data available in the literature, are incapable of providing absolute values for a pH - activity curve for contractility. It is possible, however, to construct such a curve on a relative basis.

6. The decrease in excitability produced by increasing CO_2 partial pressures is reflected in a decrease in the dynamic constant of excitability, k, as defined by Blair.

7. The effect of CO_2 on k can be analyzed in a fashion similar to the one used for the CO_2 effect on the tetanus tension. The

analysis shows that the excitability is affected without the participation of a "fixed intracellular" base.

8. The decrease of tetanic tension by high CO_2 partial pressures is not a secondary effect produced by changes in, or failure of the excitable system.

9. The attenuation of the tetanic tension produced by decreases in the pH shows a reasonable correlation with the effects of pH on contracting, glycerol-extracted muscle-fibers.

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