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STUDIES ON THE LOCALIZATION AND MECHANISM OF ALKALINE METAL ACTIVATION OF PROTEIN SYNTHESIS

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SUMMARY

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1. The in vitro activation of rat liver protein synthesizing systems by alkaline metals and ammonium ions occurs in a bimodal fashion with respect to the concentration. In microsomal systems the two K^+ optima lie between 80-110 and 155-165 mM.

2. Detailed studies with K^+ show that the relative magnitudes of the two optima change when different energy regenerating systems are employed. With endogenous pyruvate kinase (EC 2.7.1.40), the higher optimum is more apparent.

3. The bimodal response of the incorporation process per se, which is the final expression of a multiple component dependency on K^+, for the most part involves steps concerned with the interaction between amino acyl S-RNA and the ribosomal complex.

4. When this latter reaction is studied using microsomes, four to six individual peaks of K^+ stimulation are discernible. Aside from the technical aspects, the number of optima depends on prior activity of the microsomes.

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5. With E. coli ribosomes, multiple K^+ optima are seen for amino-acid incorporating systems which employ either native or synthetic messenger RNA.

6. Indications are that these multiple optima are an expression of a ribosomal property which is altered by prior activity.

7. The findings are discussed in the light of the presently expressed concepts concerning the structure of the functioning polysome.

INTRODUCTION

The incorporation of amino acids into precipitable protein by hepatic cell-free protein synthesizing systems is known to require K^+ for optimum activity[1-3]. The requirement of potassium in this process can be replaced in part by rubidium or to a lesser degree by sodium[1]. The probable physiological importance of potassium in the process of protein synthesis has been alluded to in a previous paper[4]. In earlier reports[1,2], the potassium optimum was not narrowly defined and seemed to change, depending on the physiologic state of the animal prior to sacrifice or as a consequence of the methodology. It had also been established that potassium affects more than one component of the protein-synthesizing mechanism[2,5,6].

The aim of the present work is to define in more detail, the K^+ dependence of an in vitro protein synthesizing system to facilitate physiologic studies.

Additional experiments were designed to obtain further information on the site of action of K^+ as an activator of ribosomally oriented

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protein synthesis, and possibly to delineate the role of alkaline metals in this process.

METHODS

Animals

Male Sprague-Dawley rats weighing between 200-250 grams were supplied by Simonsen Laboratories, Gilroy, California.

Preparation of tissues

Rats were killed by decapitation and bled for 20 seconds. Liver homogenates were prepared in 2.50 volumes of a medium containing 0.25 M sucrose, 0.010 M $MgCl_2$, 0.035 M Tris buffer, pH 7.8. The homogenates were centrifuged in an International PR-2 centrifuge at 14,000 x g for 9 minutes. The middle portion of this mitochondria-free supernatant was used in the incubations. All preparations were kept cold (0°-4°) until the time of incubation.

Rat liver ribosomes were prepared according to Rendi and Hultin[7] and suspended in medium. Homogenates low in sucrose (0.15 M) were used to prepare cell sap by centrifugation at 105,000 x g for 55 minutes in a Spinco Model L centrifuge at 0°.

The pH 5 supernatant was prepared from this cell sap as were the washed microsomes. For these, 5 ml of mitochondria-free supernatant was gently layered over 6.5 ml of medium containing 0.3 M sucrose. The microsomes were then sedimented at 105,000 x g. They were suspended in 5 ml of the 0.15 M sucrose medium.

Other preparations

S-RNA labeling with either [1-C^{14}] tyrosine, leucine, or C^{14} algal protein hydrolysate was done according to Moldave[8].

Carbamyl kinase (EC 2.7.2.2) was prepared from Streptococcus Fecalis, strain D-10 according to the method of Mokrasch, et al.[9].

E. coli B ribosomes and cell sap were prepared according to the method of Lengyel, et al.[10] from E. coli grown by the method of Littauer and Eisenberg[11]. E. coli S-RNA was prepared according to the method of Zubay[12].

Chemical determinations

Potassium and sodium concentrations of the mitochondria-free supernatant or of completed incubation systems were determined on properly diluted aliquots in a Baird Atomics KY-1 flame spectrophotometer having a Lithium internal standard. In the case where the incubation systems contained Li^+, K^+, determinations were done on a Beckman DU spectrophotometer with flame photometric attachment. Protein concentration was determined by the method of Lowry, et al.[13].

DETERMINATION OF RADIOACTIVITY

After separation of the particulate and soluble fractions, the proteins were precipitated and extracted as previously described[4]. The protein samples were pressed onto copper planchets having a diameter of 0.85 centimeter. The samples were then counted in a Tracerlab Omni-Guard low background gas flow counter. The background of this machine is less than 1 count per minute. Radioactivity was expressed as dpm/mg of

protein by the employment of a C^{14} methacrylate standard for calibrating the counter, and by the employment of the equations for self-absorption corrections suggested by Muramatsu and Busch[14]. The radioactivity of the labeled S-RNA was determined in similar planchets using infinitely thin samples. These were compared to an empirical curve obtained with standardized barium carbonate C^{14}. Sufficient counts were taken to insure less than 4 percent error in the case of the cell sap and less than 2 percent error in the case of the more active particles.

Chemicals

Na_2ATP, Na_2GTP, disodium creatine phosphate were purchased from Cal Biochem, Los Angeles. The PEP·TCHA* and creatine kinase (EC 2.7.3.2) were products of Boehringer Gmbh, Mannheim, Germany. Carbamyl phosphate dilithium was obtained from Nutritional Biochemicals, Cleveland. [1-C^{14}] valine, leucine and tyrosine and [U-C^{14}] phenylalanine, as well as algal protein hydrolysate, were obtained from New England Nuclear, Waltham. Polyuridylic acid was obtained from Miles Laboratories, Elkart, Indiana.

RESULTS

The use of PEP·TCHA offered an opportunity to establish to a high degree of accuracy the K^+ optimum of a liver protein synthesizing system relatively free of other alkaline metals. Initial experiments with liver

*Abbreviations: PEP, PEP·TCHA, phospho-enol pyruvate and its tricyclohexylammonium salt.

systems using K^+ as an activator indicated two optima. In view of this finding the chloride salts of the common alkaline metals and ammonium ion were re-investigated. The results of such a series are illustrated in Fig. 1. In a group of experiments shown here, the mitochondria-free supernatants from six rat livers were pooled to establish relationships between the different ionic species. In other experiments not illustrated, the different ions were tested using single rat livers. Two optima were evident so we can say that they are not a reflection of two differing physiologic states of the different rat livers. From this family of curves, it can be seen that the ions tested, with the possible exception of Li^+ and Na^+, activate protein synthesis in a bimodal fashion and extrapolation to the zero concentration affirms to an absolute requirement for an ion of this series. Microsomal radioactivity for the most part indicates two optima although low activity tends to obscure the curve form for Li^+ and Na^+.

The ability of the different ions to stimulate the incorporations as determined by the level of microsomal radioactivity for either optimum has the relationship $NH_4^+ > K^+ > Rb^+ > Li^+ > Na^+ > Cs^+$. Generally speaking, the two optima exhibit various degrees of overlap with Rb^+ and Cs^+ showing rather complete separation. Lithium, which is the first metal in the series, shows such a high degree of overlap that the algebraic summation imparts greater activity in the mid-portion of the curve than is exhibited by Na^+. With cell sap, the relative capacities of the various ions to solubilize protein have the following relationship $(NH_4^+, K^+) > Rb^+ > Na^+ > Li^+ > Cs^+$. In the mid-range region K^+ induces

a greater release than NH_4^+. The bimodal response, however, for the most part is not as clearly defined by cell-sap radioactivity.

With PEP·TCHA and only endogenous pyruvate kinase as an energy regenerating system, the K^+ activation curve for normal fed male rats is illustrated in Fig. 1. In fasted rats the second optimum is not so prominent. Such a curve can be seen in Fig. 2. For both fed and fasted rats the analysis of 25 such curves obtained from single rat livers indicates that the two optima lie between 80-110 mM and 155-165 mM. These K^+ concentrations were obtained by direct flame photometric analysis of aliquots of the incubation mixture. These were checked by summing the known additions. In this latter case, the K^+ content of the mitochondria-free supernatant was necessarily obtained by flame photometry. In Table I, the protein, K^+, and Na^+ content of a standard incubation system without added K^+ is given.

Although homogenization was done using Tris buffered medium (pH 7.8), the pH was read at 5°. The actual pH at an incubation temperature of 37° was, in fact, 7.0. This pH seemed more realistic when NH_4^+ was studied. However, even with K^+ we found little pH dependency over the range in question.

K^+ dependency and the energy regenerating system

Sachs[3] has already established that in vitro systems of this type still require K^+ independent of the needs of the energy regenerating systems. For this reason it was initially presumed that the bimodal response seen here could represent a potassium dependency inherent in the protein synthesizing machinery coupled with the K^+ requirement of

the pyruvate kinase[15]. This presumption was tested by using carbamyl kinase-carbamyl phosphate or creatine kinase-creatine phosphate, for which no K^+ requirements have been reported, as energy regenerating systems[1,2,4]. A typical experiment comparing creatine phosphate and PEP energy regenerating systems is seen in Fig. 2. Noticeably, two optima still exist. However, the 155-165 mM peak is no longer predominant. In this series valine was used; however, the dual optima are discernible with leucine, alanine, and tyrosine as well.

Contribution of the microsomal membrane

In Fig. 3, with rat liver ribosomes and carbamyl kinase-carbamyl phosphate as an energy regenerating system, the dual optima are even more pronounced. It thus can be concluded that the basis of this dichotomy does not reside in the microsomal membrane.

The very high incorporation of this type of system is quite remarkable.

Multiple component K^+ dependency in E. coli

Experiments with E. coli cell-free systems demonstrated two major optima with leucine and at least two with tyrosine and phenylalanine. These and other experiments with E. coli will be discussed in more detail below.

K^+ dependence of transfer of C^{14} amino-acyl S-RNA to rat liver microsomes

In preliminary experiments with both tyrosine and leucine labeled rat liver S-RNA, it was first determined that the incorporation of

radioactivity into microsomal, and subsequently soluble protein, was, in fact, K^+ dependent. Very little transfer to microsomal protein took place in low K^+ and radioactivity in the soluble protein fraction was nonexistent. With near optimal amounts of K^+, transfer was much more vigorous and measurable solubilizations took place, particularly between 10 and 15 minutes.

Although transfer to ribosomal preparations was also observed, it was decided to study washed microsomal preparations since one of the two required transferases is concentrated in the microsomal membrane[16].

It has been reported[6] that tyrosine activating enzyme is K^+ dependent; likewise using E. coli systems, the transfer step studied here has also been reported to have a K^+ optimum at 70 mM.[5] Therefore, one of the optima in a complete protein synthesizing system could be a reflection of the K^+ sensitivity of an earlier activation reaction. Furthermore, only one of the 20 amino acid activating enzymes need be so affected, since in the system used here, participation of all 20 amino acids would have to occur for the formation of measureable amounts of precipitable protein.

Thus, we endeavored to by-pass the complications presented by the K^+ dependency of tyrosine activation and possibly of other amino acids. S-RNA tagged with radioactive tyrosine was used. However, it is important to note that these preparations contain the full spectrum of amino-acyl S-RNA.

A K^+ dependency curve for the transfer of tyrosine-acyl S-RNA is illustrated in Fig. 4. The saw-tooth nature of the curve was at first

disregarded. It was concluded to be real when it was reproducible not only with a different method[8] but also with leucine tagged S-RNA. Characteristically, four peaks are seen with K^+ values centering about 70, 100, 120, and 160 mM. For reasons which will become clear shortly, it is probable that the 70 mM peak is a composite of two other distinct optima.

Since multiple optima were also observed with leucine-acyl S-RNA, the activation process was excluded as being contributory to those observed here. We therefore endeavored to define these optima in more detail using highly radioactive S-RNA labeled with algal protein hydrolysate and by determining a greater number of points on the curve. At the same time, alterations induced by prior protein-synthetic activity of the microsomal preparations were studied. Such an experiment is illustrated in Fig. 5. In one case, the mitochondria-free supernatant was pre-incubated with all constituents necessary for protein synthesis including K^+ at 160 mM. Both sets of microsomes were then separated through a sucrose gradient and then reacted with pH 5 supernatant and radioactive S-RNA.

In the preparation not subjected to prior incubation, six optima are distinguishable with approximate values of 55, 80, 100, 125, 160, and 190 mM. A confluence of the 55 and 80 mM peaks most probably gives rise to the 70 mM optimum previously described for tyrosine labeled S-RNA.

Concerning peak 6 at 190 mM K^+, such an optimum has, on rare occasion been observed in microsomal preparations performing all the cytoplasmic protein-synthetic processes. Its instability is amply demonstrated by its absence in preparations which had been pre-incubated

under conditions conducive to protein synthesis. In addition, pre-incubation at the supposed optimum of the 5 peak (160 mM) decreases all optima to a similar degree when they are expressed in terms of specific activity. Quantitatively, it is most likely that transfer is greatest at 160 mM. This conclusion is supported by experiments which show a K^+ dependent, energy requiring release of protein and RNA from microsomes during protein synthesis[17]. This release occurs in quantities sufficient to be determined by chemical means (cf. 2).

K^+ requirements of E. coli systems using either native or synthetic messenger RNA

As mentioned earlier, E. coli cell-free systems employing ribosomes likewise exhibited a multiplicity of K^+ optima. Four areas of stimulations were usually distinguishable. This was particularly true with tyrosine. However, the possibility of the involvement of tyrosine activating enzyme in these phenomena could further be tested in E. coli systems by the use of polyuridylic acid as a messenger for the production of polyphenylalanine. Thus a system which involves only reactions of phenylalanine could be studied with respect to K^+ requirements.

From Fig. 6, it can be seen that a system of this type displays at least three discrete areas of stimulation. This was observed with freshly prepared ribosomes. In systems where the ribosomes had been stored frozen for some time (2-4 weeks), one broad peak with an optimum at 70 mM was seen using polyuridylic as a messenger.

DISCUSSION

The use of the metal-free salt of PEP affords the opportunity to control with great precision the K^+ content of *in vitro* protein-synthesizing systems. It is, of course, true, that pyruvate kinase is K^+ dependent but the use of this type of system for physiologic comparisons has been widespread; therefore a more detailed description of this system is in order.

A comparison of the three types of energy regenerating systems shows that to a large degree these control the observed rate of protein synthesis. This is due in part to chemical differences, such as the presence of Li^+ or Na^+ (cf. 1), and in part to differing rates of regeneration of ATP. The turnover number as well as the quantity of enzyme present are factors in this latter effect. Thus, although pyruvate kinase has a turnover number much greater than creatine kinase[18], only endogenous quantities of the former enzyme were used. Reviewing Fig. 2, it can be seen that using pyruvate kinase one may conceivably observe changes in protein synthesis which are in fact alterations in this crucial enzyme.

On the other hand, it is possible that the optimum at the low K^+ concentration normally predominates over that at 160 mM. If using PEP as an energy regenerating system alters this relation, one might rightly expect an apparent shift since pyruvate kinase in itself has K^+ requirements in the 155-165 mM range[15]. Such shifts have been reported by

Hultin, et al.[1], for in vitro protein synthesis and, more specifically, for the release of ribosomal protein[2].

However, with particular reference to such experiments as illustrated in Fig. 2, these shifts may also be due to the great differences in the rates of activity of the two systems. It is known that polysomes break down sequentially into smaller oligosomes and single ribosomes during the course of polypeptide formation[19]. As we shall discuss presently, it is probable that oligosomes have K^+ requirements relative to their aggregate size. It would then follow that K^+ requirements would change relative to the preceding rate of polypeptide formation.

As to the localization of the K^+ requirements for protein synthesis, it would appear that there is a multiple component dependency. It is in the nature of these components that the optima differ in such a manner that the total expression is bimodal. There is at least one K^+ dependent reaction in the activation of the amino acids[6], however, when this step is by-passed and its obscuring tendencies are removed, a number of peaks attributable to ribosomes become discernible. The existence of multiple optima in E. coli was demonstrated by a different approach which excluded the possible involvement of tyrosine activation. Furthermore, these E. coli experiments showed that a multiple response is observable when only reactions of one amino acid are involved; and ageing of ribosomes alters the response pattern.

The recent series of studies which demonstrate the involvement of a polysome as the functioning apparatus for the formation of

polypeptides appears to have some relation to the multiple optima observed here, particularly the five or six which are most clear when the transfer reaction is isolated.

Thus, although Henshaw, et al.[20], have shown that monomer liver ribosomes obtained from cell sap are inactive in vivo as well as in vitro unless synthetic messenger RNA is present, single ribosomes obtained from the endoplasmic reticulum have the capacity to incorporate amino acids[21]. The basis of this difference is judged to be the presence or absence of messenger RNA.

Therefore, although pentamers appear to be the predominant active form in liver as well as reticulocytes[19,22,23,24], biochemical evidence[19,21,23] as well as theoretical considerations[22,23,24] impart some activity to monomers and other intermediate forms as well as the larger aggregates.

If the K^+ requirement of an active oligosome is proportional to the number of sub-units of which it is composed, then discrete changes in polypeptide formation relative to increasing K^+ levels could be expected.

For the sake of the discussion, it will be assumed that the 55 mM peak represents the monomer optimum and so on up to the hexamer optimum at 190 mM. Accordingly, pre-incubation should cause a decrease or loss of the larger aggregates but would not necessarily lead to an accumulation of labeled monomer forms since these are being expelled into the soluble fraction along with some of the synthesized polypeptide. The uniform decrease in transfer activity for all remaining optima suggests

that all forms are equally active at 160 mM. This implies a certain regulatory function of the microsomal membrane with regard to the ionic concentration about the functioning ribosomal complex.

As to the manner by which alkaline metals and NH_4^+ act to stimulate protein synthesis, Ling[25] has attempted to explain many of the biological effects of these ions by a theory involving fixed charges. Warner, et al., believe the functioning polysome to be linearly extended[21,24]. Possibly then these ions activate polypeptide formation by causing an elongation of the oligosome, the concentration of ion required being proportional to the length of the oligosome.

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TABLE I

COMPOSITION OF STANDARD RAT LIVER INCUBATION SYSTEM WITH ALL ADDITIONS BUT MADE UP TO VOLUME WITH WATER. FINAL VOLUME 2.34 ml. NUMBER OF CASES IN BRACKETS.

Protein, mg (8)		K^+, mM (25)	Na^+, mM (25)
Cell Sap 43.9 ±1.1* Microsomes 18.1 ±0.9	62.0	13.2 ±0.2	5.3 ±0.2

*S.E.

FIGURE LEGENDS

Fig. 1. Alkaline metal activation of a rat liver protein synthesizing system. Mitochondria-free supernatant was prepared in 2.50 volumes of KCl-free medium as described in Methods. Each incubation tube contained 20 μmoles of PEP·TCHA, 2 μmoles of ATP Na_2, 0.4 μmoles of GTP Na_2, 0.1 μmole of [1-C^{14}] valine having 0.588 μC of radioactivity, 1.4 ml of the mitochondria-free supernatant having the composition listed in Table I, and additions of the pertinent ions in amounts to give the above concentrations in a final volume of 2.34 ml. The incubation period was 10 minutes at 37°.

Fig. 2. Comparison of endogenous pyrovate kinase and exogenous creatine kinase based energy regenerating systems on the K^+ activation curve of microsomal liver protein synthesizing systems. Preparations were from a single fasted liver. All conditions were exactly as in Fig. 1 except that in one case, creatine phosphate Na_2 and the addition of 100 μgm of creatine kinase per tube were substituted for PEP·TCHA. Broken lines, microsomes; solid lines, cell sap. Filled symbols are creatine phosphate curve. Cell sap scale on right; microsomes on left.

Fig. 3. K^+ dependence of amino acid incorporation using liver ribosomal preparations and carbamyl phosphate-carbamyl kinase to regenerate ATP. The system consisted of 0.7 ml of ribosomes (10 mg protein) dissolved in low sucrose media (see Methods) and 0.7 ml of cell sap. Other additions were as in Fig. 1 except that carbamyl phosphate Li_2 was substituted for PEP·TCHA and 0.05 ml of a highly purified carbamyl kinase solution was

added. Broken line with dark symbols, ribosomal incorporation. Cell sap scale on right; ribosomes on left.

Fig. 4. K^+ dependence curve for the transfer of [1-C^{14}] tyrosine labeled S-RNA radioactivity to microsomal and soluble protein. Each reaction tube contained 0.4 μmoles Na_2GTP, 2 μmoles Na_2ATP, 20 μmoles creatine phosphate, 100 μgm creatine kinase, 0.7 ml of pH 5 supernatant (12 mg protein), 0.7 ml washed microsomes (8 mg protein), 0.12 mg tyrosine labeled S-RNA having 4300 dpm with the indicated K^+ concentrations in a final volume of 2.3 ml. Incubation period was 10 minutes at 37°. Broken lines with dark symbols, microsomal incorporation. Microsomal counts on left scale; soluble protein on right.

Fig. 5. K^+ dependence curve for the transfer of amino-acyl S-RNA radioactivity using S-RNA labeled with algal protein hydrolysate. All conditions of the incubation are as in Fig. 4 except that 0.06 mg of labeled S-RNA having 44,000 dpm was used. Mitochondria-free supernatant was mixed with creatine phosphate energy regenerating system, GTP, ATP, and KCl (160 mM). One portion was incubated at 37° for 5 minutes and the other was kept on ice. The microsomes were then obtained and transfer capacity was studied. Except for the prior treatment of the microsomes all conditions were identical in both sets of reaction vessels. Filled symbols: pre-incubated microsomes and soluble protein fraction. Square symbols are microsome specific activity with scale on left. Circles are soluble protein specific activity with scale on right.

Fig. 6. Comparison of fresh E. coli protein synthesizing systems using endogenous messenger RNA or polyuridylic acid messenger. Each tube contained 111 μmoles Tris buffer (pH 7.8); 39 μmoles $MgCl_2$; 33 μmoles

mercaptoethylamine; 2.7 μmoles ATP Na_2; 0.66 μmoles GTP Na_2; 3.6 μmoles creatine phosphate Na_2; 1.3 μgm creatine kinase; 9 mg E. coli S-RNA; ribosomes (8.4 mg as protein); supernatant dialyzed against H_2O (11 mg as protein); 0.27 μmole each of 20 amino acids (cf. 12); 0.5 μC [U-C^{14}] phenylalanine (297 μC/μmole); plus the indicated KCl concentrations. One set of tubes (filled symbols) was pre-incubated 15 minutes at 37° in bulk before addition to tubes containing the KCl, S-RNA, radioactive phenylalanine, and 240 μgm of polyuridylic acid. Both sets of tubes were subsequently incubated for 10 minutes and extracted as previously. Square symbols are ribosomal activity; open symbols represent system employing native messenger RNA. Left Scale: pre-incubated ribosomes and cell sap. Right Scale: Non-pre-incubated using endogenous messenger RNA.

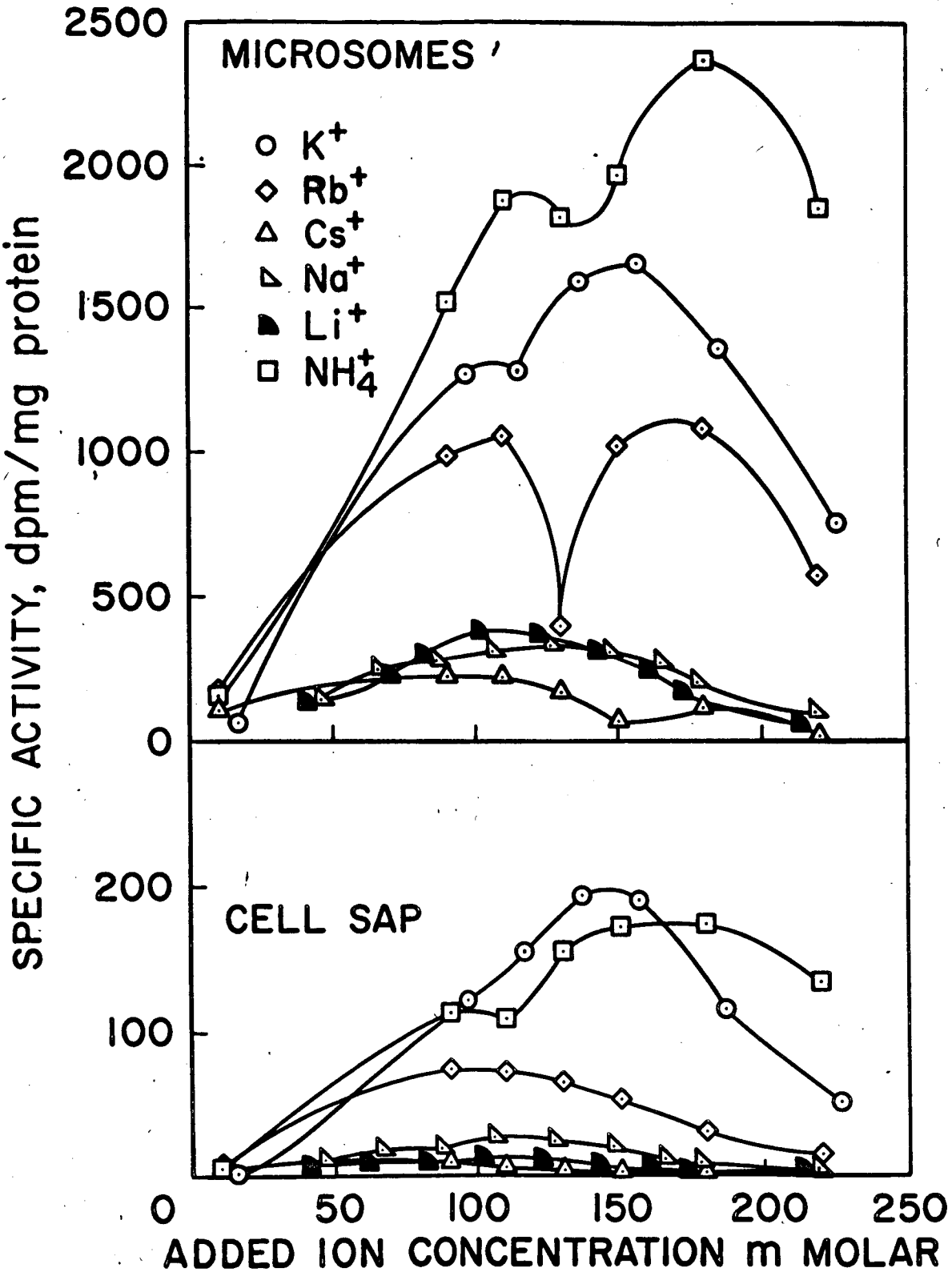

Figure 1.

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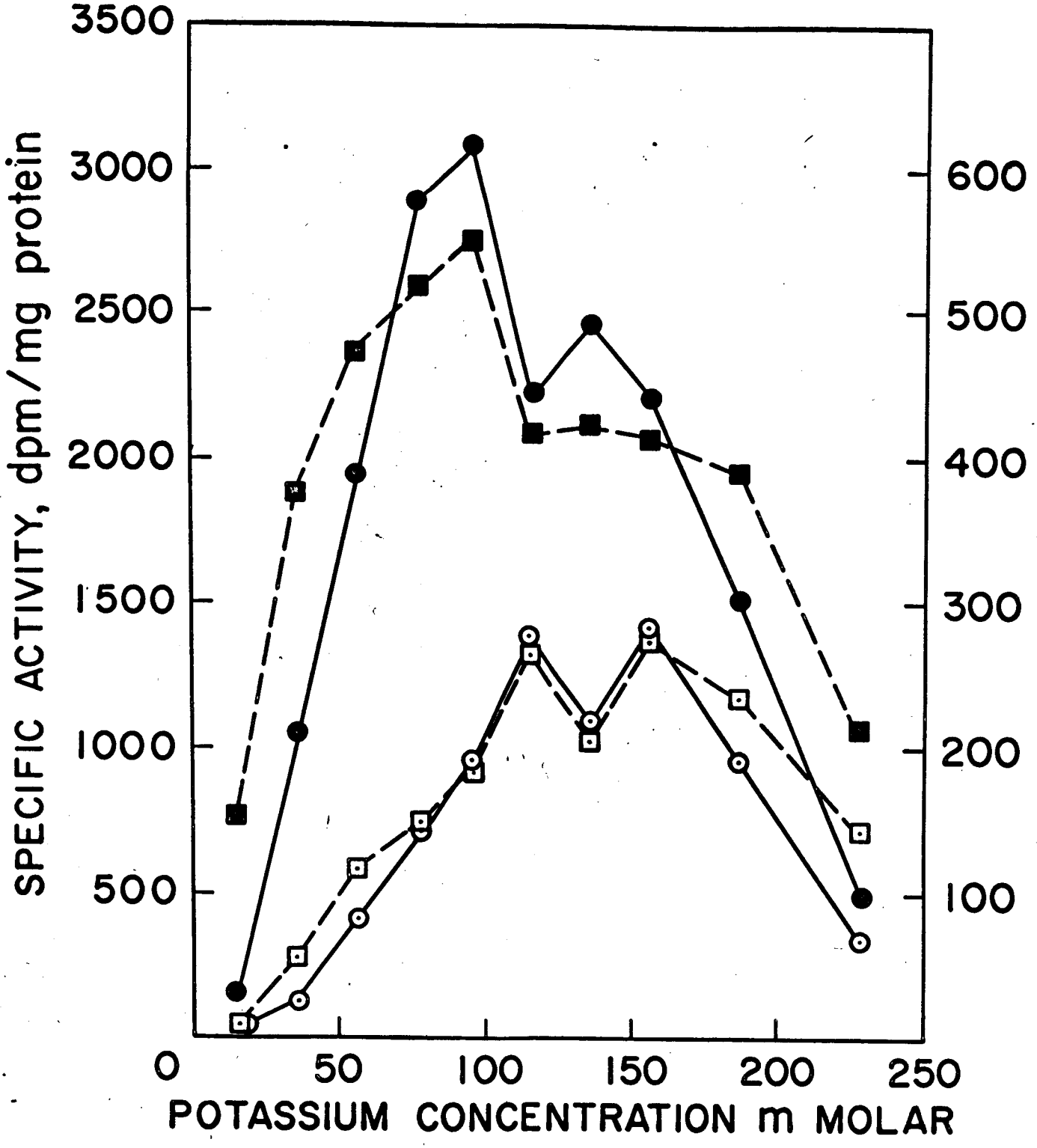

Figure 2.

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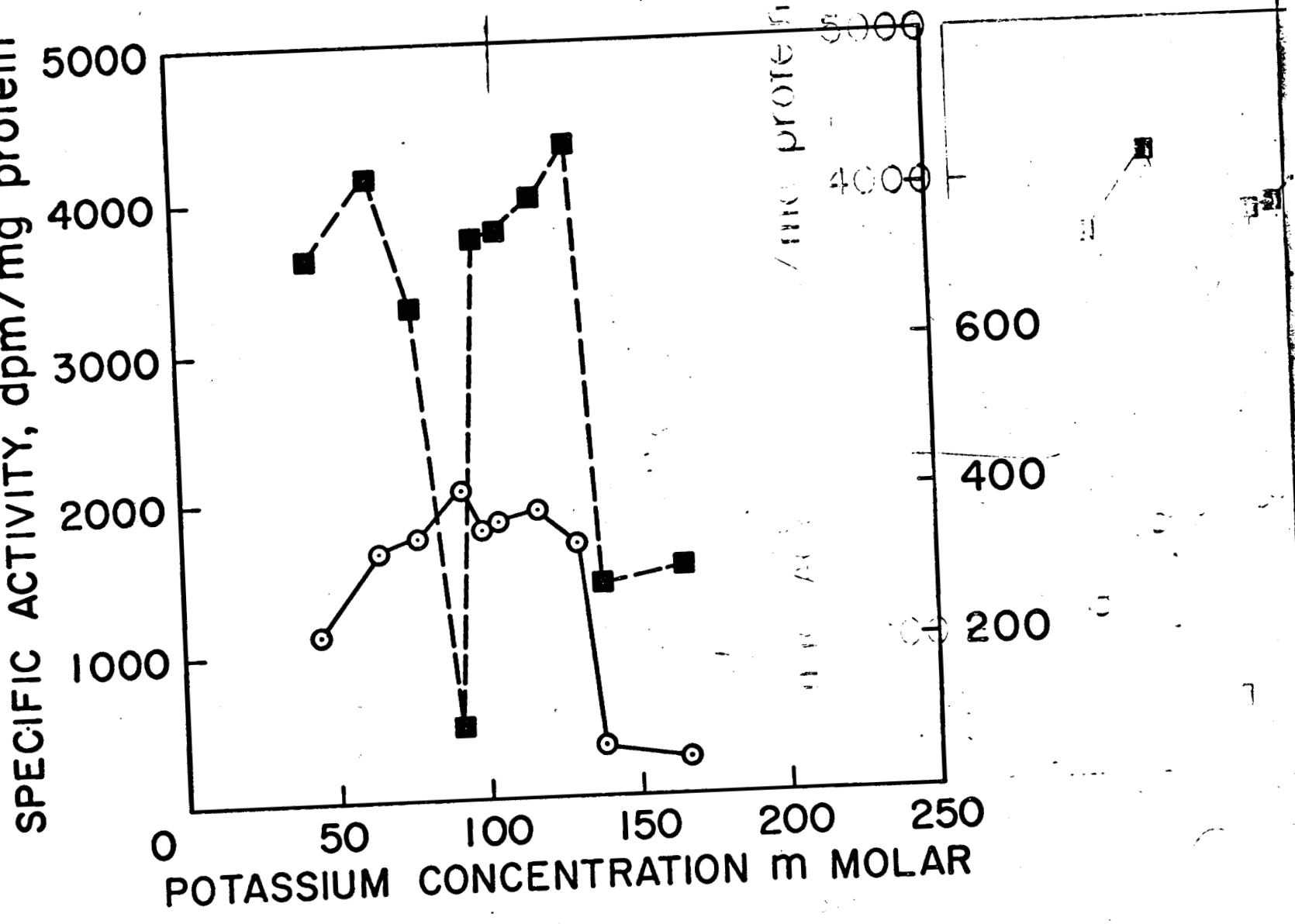
SPECIFIC ACTIVITY, dpm/mg protein
5000
4000
3000
2000
1000
0
50
100
150
200
250
POTASSIUM CONCENTRATION m MOLAR
600
400
200

Figure 3.

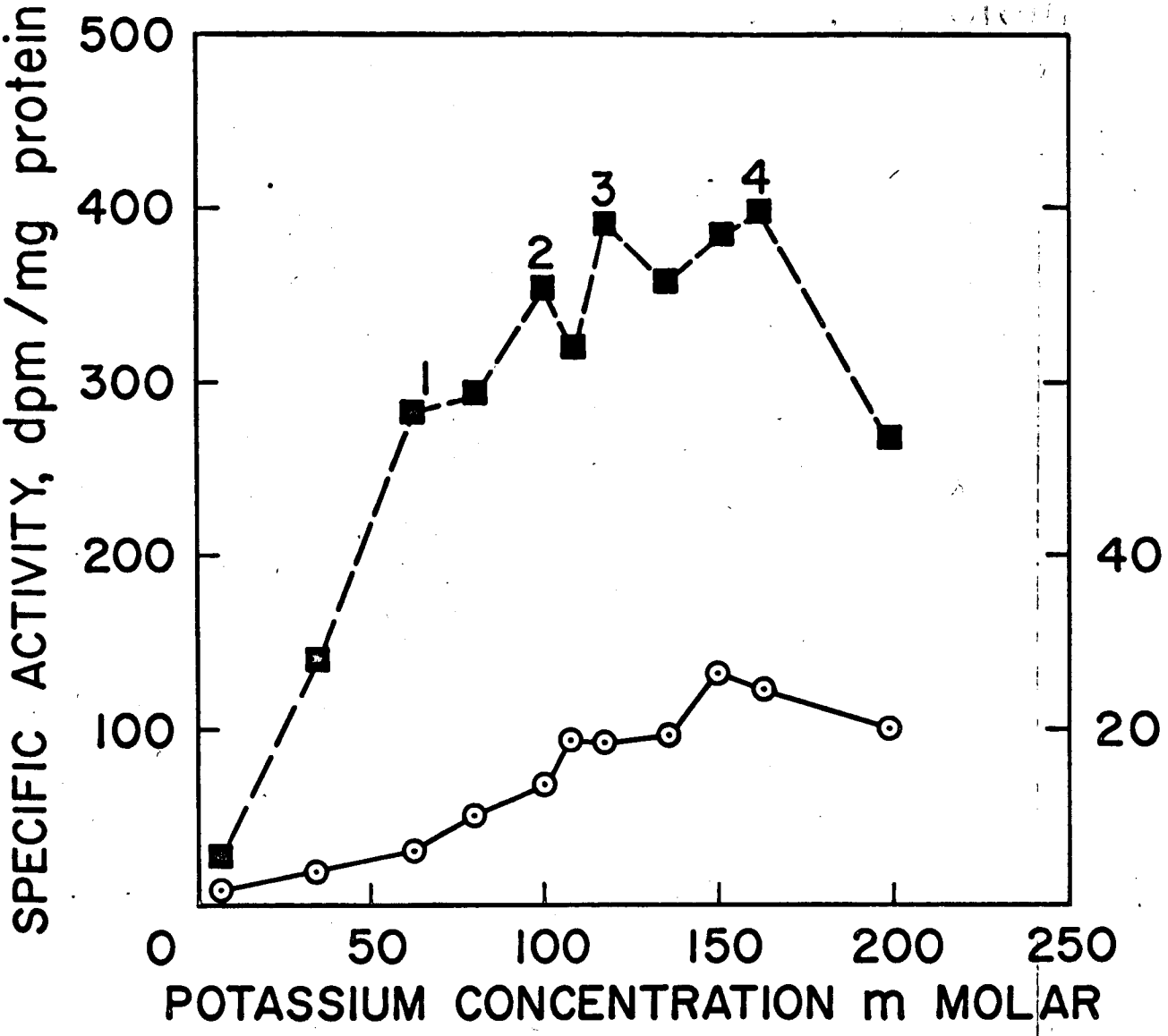

Figure 4.

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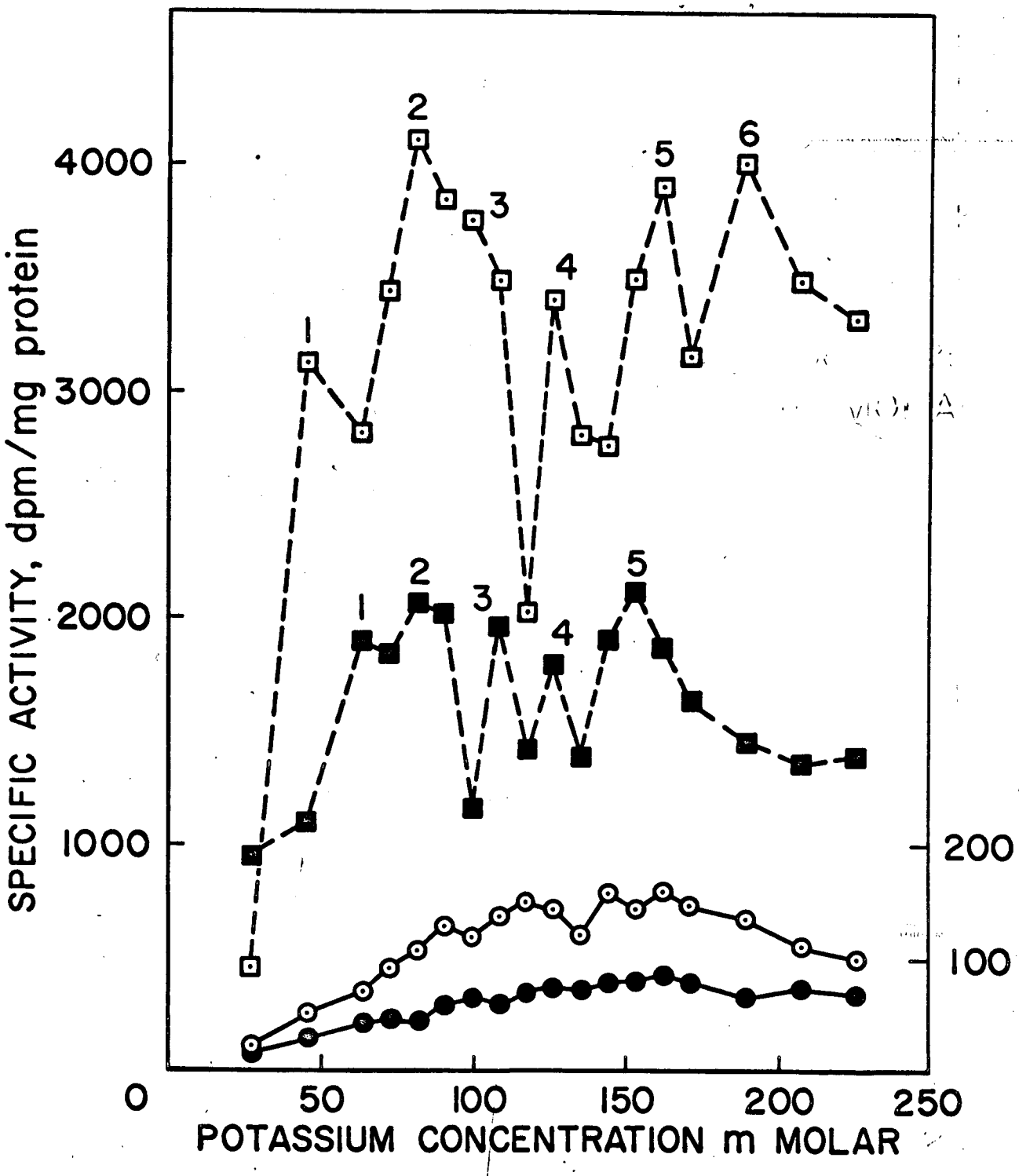

Figure 5.

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POTASSIUM CONCENTRATION m MOLAR

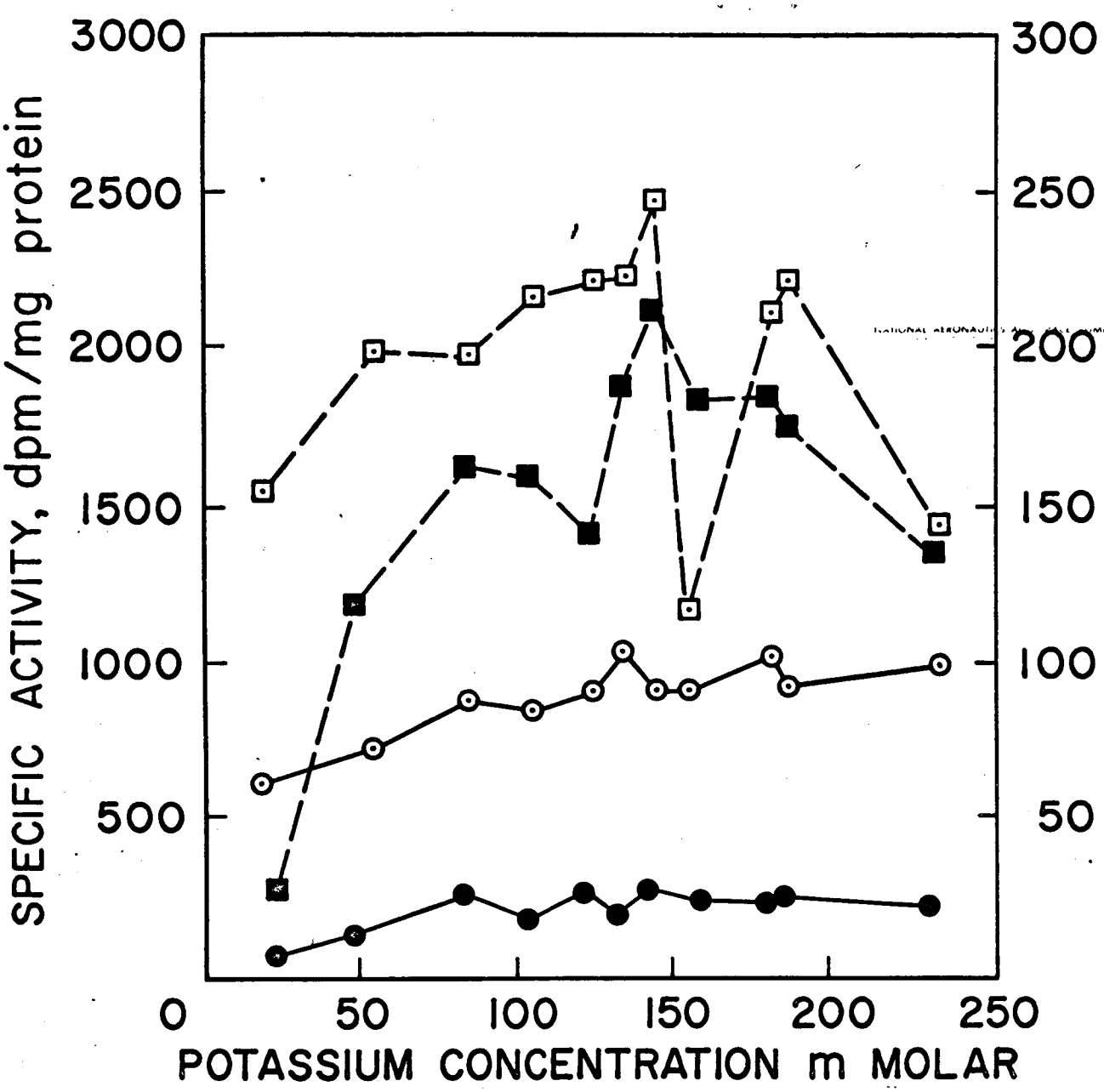

Figure 6.

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