CYCLIC UPTAKE OF CALCIUM-45 BY DIVIDING SEA URCHIN EGGS

by

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INTRODUCTION

The present study was undertaken to provide direct data on the uptake of calcium by dividing eggs of the sea urchin, and to relate this, as far as possible, to specific mitotic events.

The role of calcium in cell division has been a subject of interest for many years, the earlier ideas stemming largely from the experiments and studies of L. V. Heilbrunn, who stressed the role of calcium in initiating protoplasmic clotting or gelation (5, pp. 166-194). The relevance of this reaction to cell division consists in the finding (16) that fertilization of the sea urchin egg is accompanied by the breakdown of a calcium-proteinate gel in the cell cortex and is followed by an increase in the free calcium of the protoplasm (12). The subsequent increase in protoplasmic viscosity (4) associated with development of the mitotic spindle and asters was regarded by Heilbrunn as a calcium-induced process of protein gelation, as suggested by analogy with experimental observations on the amoeba. In this organism the viscosity of the cortical protoplasm rises markedly when exposed to solutions rich in calcium but decreases sharply when the amoeba is immersed in an oxalate solution or in a solution containing an excess

of potassium ions (6,7). More recently, Hultin has shown (9) that egg homogenates of the sea urchin prepared in the absence of calcium show a sharp increase in viscosity on the addition of calcium. Likewise, Kane and Hersh (10) obtained from sea urchin eggs a soluble protein fraction which responded reversibly to the addition of calcium by forming a gel.

Recent observations by Dornfeld and Owczarzak (2) pertaining to the effects of versene on fibroblasts grown in vitro have provided further data on the relations of divalent cations, especially calcium, to cell division. Interphasic fibroblasts respond to treatment with versene (ethylenediamine tetraacetic acid, a divalent-ion chelating substance) by cellular contraction which is followed by blebbing of the cell surface. This reaction cytologically resembles the rounding of cells that normally occurs at metaphase and the blebbing of the cell surface which characterizes anaphase. This reaction is reversible and cannot be induced by calcium or magnesium versenate, hence can be attributed to the removal of calcium (or other divalent cations) from the cell. It is also producible by exposure of fibroblasts to isotonic salt solution lacking calcium and magnesium. The versene-induced contraction, or rounding, of the cell is interpreted to reflect a breakdown of the cortical protoplasmic gel through loss

of intermolecular calcium (and/or magnesium) bonds. The blebbing of the cell surface is held to result from internal hydrostatic pressure operating between loci of the surface which have become ionically reconstructed through movement of free ions from the cell interior to the surface. The hydrostatic pressure arises from osmotic uptake during prophase (1). Cells continuously perfused with versene cease bleb formation after about two hours, when the internal store of divalent ions has been depleted, but addition of calcium to the environment re-establishes bleb formation and finally restores the interphasic cell form. Cells already rounded in late prophase at the time of versene application significantly do not respond by bleb formation. At this time the mitotic spindle is being formed, and this proteinaceous structure draws the divalent ions assumedly required for its formation from the exterior environment and cellular cortex - hence the contracted state of metaphase cells. Also, blebbing cannot occur until such ions are no longer required by the spindle. This normally happens at anaphase, when blebbing ("anaphase bubbling") is autonomous, and before which it cannot be induced by versene; versene, however, will prolong this phenomenon, as in interphasic cells. From these experiments of Dornfeld and Owczarzak emerges the picture that calcium

(and possibly magnesium) is integrally involved in cell division, specifically in the changing properties of the cellular surface and in the formation of internal gel structures.

Hanson (data from research in progress) has discovered that versene will quantitatively block mitosis in sea urchin eggs. If a critical concentration of versene is added to fertilized eggs in calcium-free sea water prior to metaphase, no spindle forms and the cells do not divide. However, when restored to regular sea water, these eggs will form spindles and divide. The time required for division of blocked eggs after removal of the reagent is approximately the same as the normal time required for division from the stage at which the versene was applied. These data are interpreted as showing the involvement of calcium (and/or other divalent ions) in synthetic processes occuring in the cell at the time of spindle formation.

The uptake and movements of calcium hypothesized on the basis of the versene experiments need to be investigated by more direct methods. Measurement of Ca^{45} uptake by synchronously dividing sea urchin eggs to determine the time and amount of uptake of calcium ions appears to offer an ideal approach.

A search of the literature on the uptake of radiocalcium by sea urchin eggs disclosed only two papers.

Rudenberg (18), studied the role of the jelly coat in calcium uptake but without reference to cell division. Hsiao and Boroughs (8) attempted to measure the uptake of radioactive calcium in unfertilized eggs, without obtaining conclusive results. No report was found on the use of radiocalcium to measure the uptake of calcium coincident to various events of the mitotic cycle and cleavage.

MATERIALS AND METHODS

Mature, fertilized eggs of Strongylocentrotus purpuratus (Stimpson), the common purple sea urchin of Oregon coastal waters, were used. Freshly collected females were induced to shed their eggs by the KCl injection method of Tyler (20). Sperm were obtained by removing the intact testes from the males and were kept undiluted until used for fertilization.

The freshly collected, unfertilized eggs were gently centrifuged at 100 x gravity for three minutes. Since the egg has an average volume of 1,260,300 cubic microns then without interspaces (packed condition) there would be approximately 800,000 eggs, with jelly coats, contained in one cubic centimeter of settled eggs. For the first series of experiments (runs 1 through 6) two cubic centimeters of settled eggs, or about 1,600,000 eggs, were suspended in 400 ml. of filtered sea water and fertilized with one cubic centimeter of dry sperm, the fertilization being performed in a 500 ml. graduate cylinder. Unless 95% of the eggs (as determined by microscopic examination of samples) formed a fertilization membrane within one minute, the eggs were discarded and a new batch started. Immediately after fertilization the jelly coats were removed by adding 8 drops of 6N HCl to the sea water and mixing thoroughly. The fertilized

eggs were then washed and decanted three times to remove the acid and most of the sperm. The volume of the egg suspension was then made up to 400 ml., and with constant agitation forty 10 ml. aliquots were measured out into separate Stender dishes. A number of check counts were made to be certain that this method guaranteed a uniform number of eggs in each of the forty samples. It was found that the variation was about 2%, indicating that the number of eggs in each sample varied between approximately thirty-nine and forty-one thousand. Before addition of the eggs, each Stender dish was provided with 0.1 ml. of filtered sea water containing a constant concentration of Ca^{45}. The estimated specific activity for the radioactive calcium was adjusted to 0.19 microcurie /mg. of calcium. The total time which elapsed from the introduction of sperm until the eggs were exposed to the Ca^{45} never exceeded five minutes.

In each run the mitotic activities were synchronous in all samples as determined by microscopic checks. Runs one through six were all made at 15.5°C.

At intervals of five minutes the contents of successive Stender dishes were transferred to rimless culture tubes, 16 x 150 mm., and immersed in an ice-water bath to stop mitotic progess. Immediately prior to this, two drops of shaken suspension had been removed

from each dish and placed into fixative (10% neutral formalin) for subsequent microscopic examination. Each sample was then decanted to a 1 ml. mark and washed by the addition of 9 ml. of filtered sea water. After five repetitions of this process, the eggs were finally left in 1 ml. of sea water, which was transferred to a standard counting vial for counting by the liquid scintillation method. Thus a series of samples was obtained representing five-minute intervals and extending over a period of three hours and twenty minutes after fertilization.

The second series of experiments (runs 11 through 21) was conducted at temperatures ranging from 12 to 17°C. In this series the eggs were handled in the manner described above but not divided into separate dishes. The eggs were allowed to develop together, with constant stirring, in a battery jar placed in a running tap water bath to control the temperature of the sea water-calcium^{45}-egg mixture. At five minute intervals, 10 ml. samples were taken from the battery jar, chilled, and subsequently washed as described previously. The number of eggs in the battery jar was in this series adjusted to provide about 16,000 in each 10 ml. sample except for run number 11 where the concentration was 20,000 eggs per sample.

In run number 14, twice the usual amount of eggs, sea water, and radiocalcium were placed in a battery jar, all the eggs coming from the same female. At five minute intervals two 10 ml. samples were taken and the run thus split as number 14 and 14B. On three occasions two runs were conducted separately but simultaneously at the same temperature, with eggs from separate females. These were pairs 16 and 17; 18 and 19; 20 and 21.

For counting in the liquid scintillation spectrometer the eggs were suspended in a thixotropic gel made as follows: to 100 ml. of tolulene was added 10 grams of naphthalene, 10 mg. 1,4-bis-2-(5-phenyloxazolyl)-benzene, 200 mg. p-terphenyl, 1 ml. glycerine, 0.8 ml. polyoxyethylene sorbitan monooleate, and 2.5 grams of thixotropic gel powder (supplied by the Packard Instrument Co., La Grange, Illinois). Fourteen ml. of this gel were used per vial, making with the addition of the eggs a total volume of 15 ml. per vial. In runs 11 through 21 the naphthalene was omitted from the gel. After thorough agitation the vials were placed in a Tri-Carb Liquid Scintillation Spectrometer using a high voltage setting of 1220 volts and a window setting from 10 volts to infinity. The counting was carried to a standard deviation of no more than 1%. An intrinsic standard was set up to measure the counting efficiency.

The small portion of each sample reserved for microscopic examination was used to determine the stage of mitosis and cleavage of the individual sample. Photomicrographic records were made for some of the runs. The samples from the second series of experiments were subjected to differential counts to determine the exact times of the 50% cleavage level.

Two runs were made using unfertilized eggs but keeping all other factors the same as those for the fertilized eggs.

EXPERIMENTAL RESULTS

Cyclic uptake and release of calcium^{45} by dividing sea urchin eggs was observed to occur in a very regular manner. In contrast, the two runs using unfertilized eggs showed no significant uptake or release of calcium during the duration of the experiments.

When the counts per minute of each sample are plotted against the time after fertilization of the eggs, a pattern of peaks of high radioactivity occurs, giving in most runs, three peaks in each division cycle. In a few runs (6 out of 16) an additional small peak occurred soon after fertilization but only those three peaks following pronuclear fusion are considered in this report. The single runs (each representing a batch of eggs from a different female) comprise numbers 1, 2, 3, 4, 5, 11, 12, and 13. Graphs of run 4 and run 13 are shown in figures 1 and 2, respectively, and are characteristic of these single runs. Graphs of two runs on unfertilized eggs are shown in figures 3 and 4. The results of duplicate samples from the same female (runs 14 and 14B) are plotted together in figure 5. Simultaneous runs (eggs simultaneously taken from two females and kept at the same temperature) include pairs 16-17, 18-19, and 20-21. Figure 6, which shows runs 18 and 19 is characteristic of these pairs.

The relation of temperature to time of occurrence for all peaks is shown in table I. At lower temperatures the peaks tend to develop more slowly. These data are included also in figure 7, together with the microscopically determined times of mid-prophase, mid-metaphase, and the 50% cleavage level.

Figure 8 shows a series of six photomicrographs, taken through phase contrast optics, depicting the mitotic stages pertinent to the experimental data.

For purposes of clarity each group of peaks will be considered separately in regard to its temporal relationship with a particular stage of the mitotic cycle.

Peak One

This peak, present in all but one run, is generally twice the magnitude of the bases between peaks. It is seen to occur at the microscopically determined period of mid-prophase (fig. 7). Mid-prophase is cytologically characterized by the first appearance of the asters and the breakdown of the membrane of the fusion nucleus; a spindle is not yet evident (fig. 8b).

Peak Two

This peak is present in every run and is about equal in magnitude to the first peak. A comparison of this peak with the mid-metaphase line (fig. 7) shows that the peak approximately follows this line along the entire

series of runs. The period of mid-metaphase (fig. 8c) is characterized microscopically by a fully developed spindle with all chromosomes on the equatorial plate and takes place, on the average, 12 to 15 minutes before anaphase (fig. 8d).

Peak Three

This peak, unlike the previous two, tends to fluctuate in magnitude but is usually somewhat higher. It follows closely the 50% cleavage level of the eggs (fig. 7), which is the time when one-half of the eggs of the microscopic sample (determined by differential counts) have formed complete cleavage furrows (fig. 8e). This peak always occurs five to fifteen minutes after anaphase.

Subsequent Peaks

This same pattern of three peaks is present in the second division cycle of the eggs (figs. 1, 2, 5, and 6). However, since division becomes more rapid and less synchronous, the peaks are closer together and not always as sharp and distinct as the corresponding peaks of the first division. The loss of synchrony makes the data of the second division less amenable to analysis.

No difference in rate of development or microscopic appearance was noted between experimental and control eggs. As a further check, a sample of each experimental batch was allowed to develop beyond the termination of

the experimental run and in each case the embryos hatched as normal swimming blastulae.

TABLE I

Peaks of Ca^{45} Uptake in Relation to Time After Fertilization and Temperature

Run No.	Temperature Degrees C.	Time After Fertilization(minutes) Peak 1	Peak 2	Peak 3
13	12.0	75	95	130
12	12.5	75	90	120
21	13.0	70	85	115
20	13.0	70	85	110
11	14.0	--	90	110
16	14.0	65	90	105
17	14.0	65	90	105
18	15.0	55	80	95
19	15.0	55	80	100
1	15.5	60	75	110
2	15.5	65	80	105
3	15.5	65	85	110
4	15.5	50	80	115
5	15.5	65	85	---
14	17.0	65	80	100
14B	17.0	65	80	100

DISCUSSION AND INTERPRETATIONS

The data presented in this study give definite evidence for cyclic uptake and release of calcium during cell division, with peaks of uptake related to specific mitotic stages.

The formation of the asters and spindle (mitotic apparatus) and the production of the cleavage furrow are essentially gel formations of endoplasmic protein. Gross (3) discusses, on purely theoretical grounds, a number of possible mechanisms for these gelations, such as a shift in the localization of calcium ions, Van der Waal's forces, hydrogen bonds, covalent bonds, and disulfide linkages. Experimental data regarding these mechanisms, as they pertain to mitotic processes, are as yet limited to the observations on calcium noted in the introduction and to the studies of Mazia and his associates on disulfide bonds. The solvent action of alkaline thioglycollate on the isolated mitotic apparatus has given evidence for the role of intermolecular disulfide bonds in aster and spindle formation (13). The prevention of disulfide bond formation <u>in vivo</u> by mercaptoethanol has tended to substantiate this (15).

On the basis of electrophoretic and amino acid analyses Mazia found the mitotic apparatus (asters and

spindle) to be composed primarily of a single, simple protein that may account for as much as twelve per cent of the total cell protein at the time of division (14). He pointed out that this protein must either have pre-existed in a soluble form during interphase or be massively synthesized at the time of cell division.

Kane and Hersh (10) extracted a soluble protein fraction from the unfertilized sea urchin egg which had many properties in common with the major protein of the mitotic apparatus. The addition of small amounts of calcium ion induced the formation of a gel in extracts of this protein. This gel can be redissolved by dialyzing off the calcium. The gelation reaction is not specific for calcium, and does occur with other alkaline earth metals; however, the minimum concentration for gelation with calcium is lower than that required for gelation with all other ions of alkaline earth metals. The concentration of calcium necessary to produce the protein "fibers" was 0.02 M. The large amount of this protein in the cell plus the calcium reaction suggested to Kane and Hersh that it may be of major importance in the structural organization of the cell, and may represent the soluble precursor of the mitotic apparatus. A recent attempt by Went and Mazia (21) to identify this protein with the major component of the mitotic apparatus,

using immunochemical methods, showed that the antibodies produced against these proteins by the rabbit are not identical. These investigators concluded that the protein of Kane and Hersh is not present in the mitotic apparatus and therefore cannot be regarded as its precursor.

Any model of spindle fiber structure which would have as its major premise the concept of protein molecules bound together via calcium bridges, must be abandoned also in the light of the data herein reported. If such a calcium bridge did exist, then there should be no release of calcium from the spindle until its dissolution. The data indicate a release of calcium at about the onset of metaphase, when the spindle is fully formed. What, then, may be the significance of the calcium uptake? It is conceivable that a protein precipitable by calcium ions is present in sufficient quantity to <u>direct</u> the formation of the spindle, in the manner of a template, without itself becoming a part of the spindle. This template protein could disintegrate as rapidly as the spindle itself becomes constructed. If regarded as a spindle precursor, this term would be used in a sense different from that employed by Went and Mazia. Microscopically the template protein would not be distinguishable from the spindle itself and would indeed

have to bear strong physical resemblance to the spindle protein. The protein of Kane and Hersh would be an excellent candidate for this substance. The first peak shown in the experimental results of this study, which appears at mid-prophase, the time of spindle formation, may represent the gelation of such a template protein.

The second peak may be suspected of reflecting a similar activity. Rustad (19), measuring by interference microscopy local mass changes in the mitotic apparatus of dividing sea urchin eggs, discovered the formation and growth of a region of high density in the interzonal area during anaphase. Although the exact nature of this region is unknown, it does, according to Rustad, represent a significant increase in the amount of protein in the interzonal area of the sea urchin spindle. The second peak, by virtue of the time of its occurence just prior to anaphase, may well be related to gelation of a template-precursor for synthesis of this interzonal body.

Similarly, the third peak may be associated with the gelation of a template protein for synthesis of new cell cortex at the time of cleavage. Although this peak occurs coincident rather than prior to the 50% cleavage level, it should be noted that cleavage

furrow formation and growth of new cortical gel is more rapid than spindle formation. The 26% increase in cell cortex occuring at this time may require a greater amount of protein synthesis than the formation of the spindle, thus accounting for the greater magnitude of this peak. The importance of gel formation during cleavage has been well established by the experiments of Marsland and associates (11).

Brief consideration should also be given to the possibility that uptake of calcium during cell division is related to its requirement as a co-factor in enzymatic reactions, especially in the breakdown of high-energy phosphates. The high amounts of calcium taken up by the cell could not, however, be accounted for by enzymatic requirements alone. Page (17) gives the concentration of calcium in sea urchin eggs as 1.90 mg. per 10^6 eggs (10^6 eggs = 0.124 gm. dry weight), equivalent to 0.047 millimols, and we may assume twice this amount to be present in the cells at peak uptake since the peaks represent approximately a doubling of the amount of calcium in the cell. Enzymatic co-factors are ordinarily required in only trace concentrations. The structural role of calcium in gel formation seems therefore to be the more likely, or at least the more prominent, function of this ion.

SUMMARY

1. The amounts and times of uptake of calcium ions by dividing sea urchin eggs were measured by using the radio-isotope calcium-45 and counting with a liquid scintillation spectrometer.

2. A total of eighteen runs were made, sixteen using fertilized eggs and two using unfertilized eggs. The fertilized eggs showed a pattern of three peaks of calcium uptake per division; no uptake of calcium was apparent in the unfertilized eggs.

3. The three peaks were correlated with the mitotic cycle as follows:

a. The first peak occurs at mid-prophase and is thought to be associated with the gelation of a template protein which directs the formation of the metaphase spindle.

b. The second peak, which occurs at mid-metaphase, is thought to be related to gelation of a template protein which directs the synthesis of the interzonal spindle, prominent during anaphase.

c. The third peak, occurring at the 50% cleavage level, is thought to be associated with gelation of a template protein responsible for the direction of the synthesis of new cell cortex at the time of cleavage.

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APPENDIX

EXPLANATION OF FIGURES

FIGURE 1

Counts per minute vs. time plot of dividing sea urchin eggs, experimental run number 4. P, prophase; M, metaphase; A, anaphase; C I, first cleavage; C II, second cleavage; 1, first peak; 2, second peak; 3, third peak.

FIGURE 2

Counts per minute vs. time plot of dividing sea urchin eggs, experimental run number 13. P, prophase; M, metaphase; A, anaphase; C I, first cleavage; C II, second cleavage; 1, first peak; 2, second peak; 3, third peak.

FIGURE 3

Counts per minute vs. time plot of unfertilized sea urchin eggs, experimental run number 6.

FIGURE 4

Counts per minute vs. time plot of unfertilized sea urchin eggs, experimental run number 15.

FIGURE 5

Counts per minute vs. time plot of dividing sea urchin eggs, duplicate runs 14 and 14B plotted together. P, prophase; M, metaphase; A, anaphase; C I, first cleavage; C II, second cleavage; 1, first peak; 2, second peak; 3, third peak.

FIGURE 6

Counts per minute vs. time plot of dividing sea urchin eggs, simultaneous runs 18 and 19 plotted together. P, prophase; M, metaphase; A, anaphase; C I, first cleavage; C II, second cleavage; 1, first peak; 2, second peak; 3, third peak.

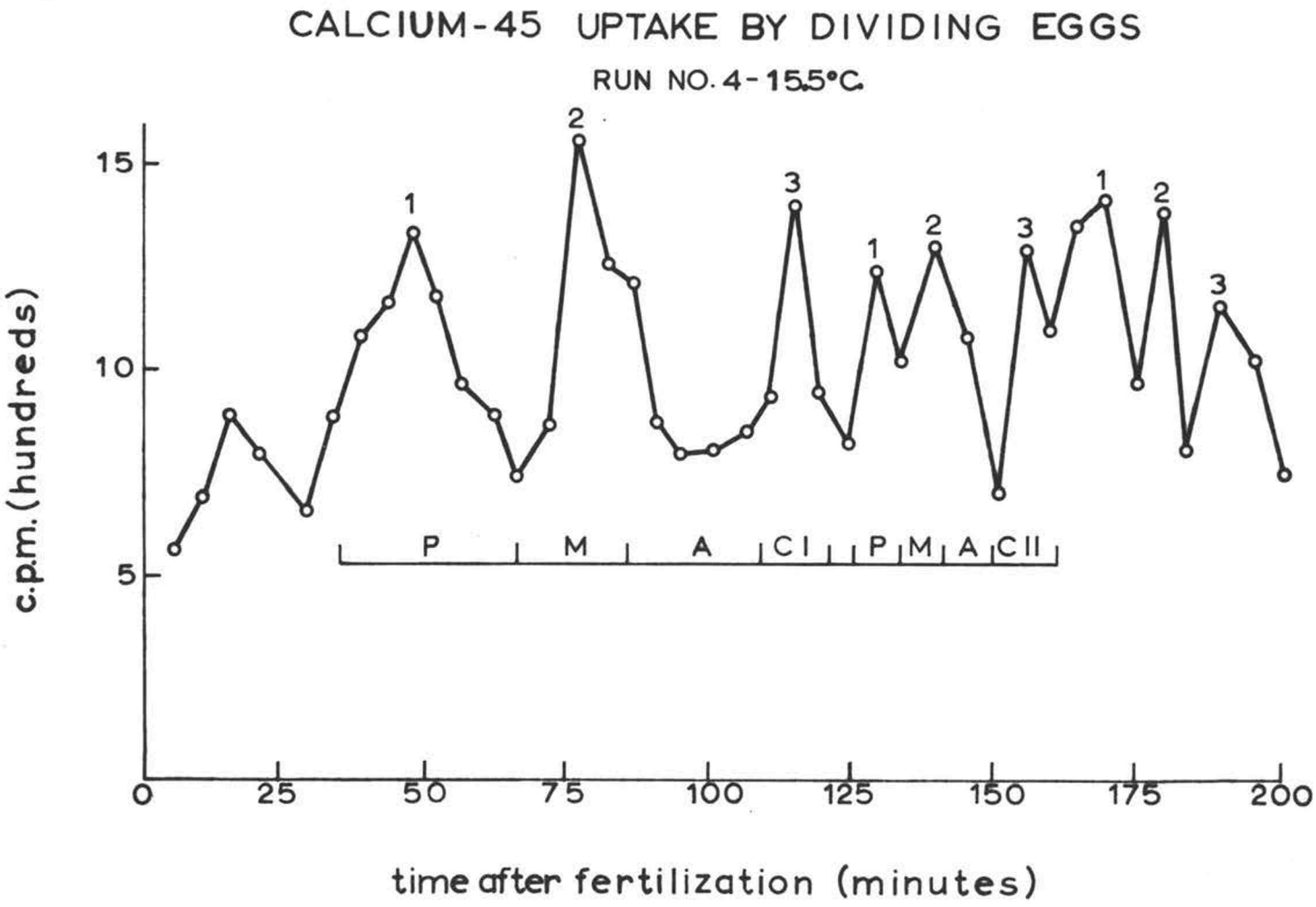
FIGURE 7

Time of occurrence of calcium peaks vs. temperature plotted with time of occurrence of mid-prophase vs. temperature, time of occurrence of mid-metaphase vs. temperature and time of occurrence of the 50% cleavage level vs. temperature. This plot shows 44 peaks obtained in 16 experimental runs.

FIGURE 8

Photomicrographs taken through phase contrast optics depicting various mitotic stages in the fertilized sea urchin egg.

Fig. 8a. Pronuclear fusion.
Fig. 8b. Mid-prophase.
Fig. 8c. Mid-metaphase.
Fig. 8d. Mid-anaphase.
Fig. 8e. First cleavage.
Fig. 8f. Mid-metaphase of the second division.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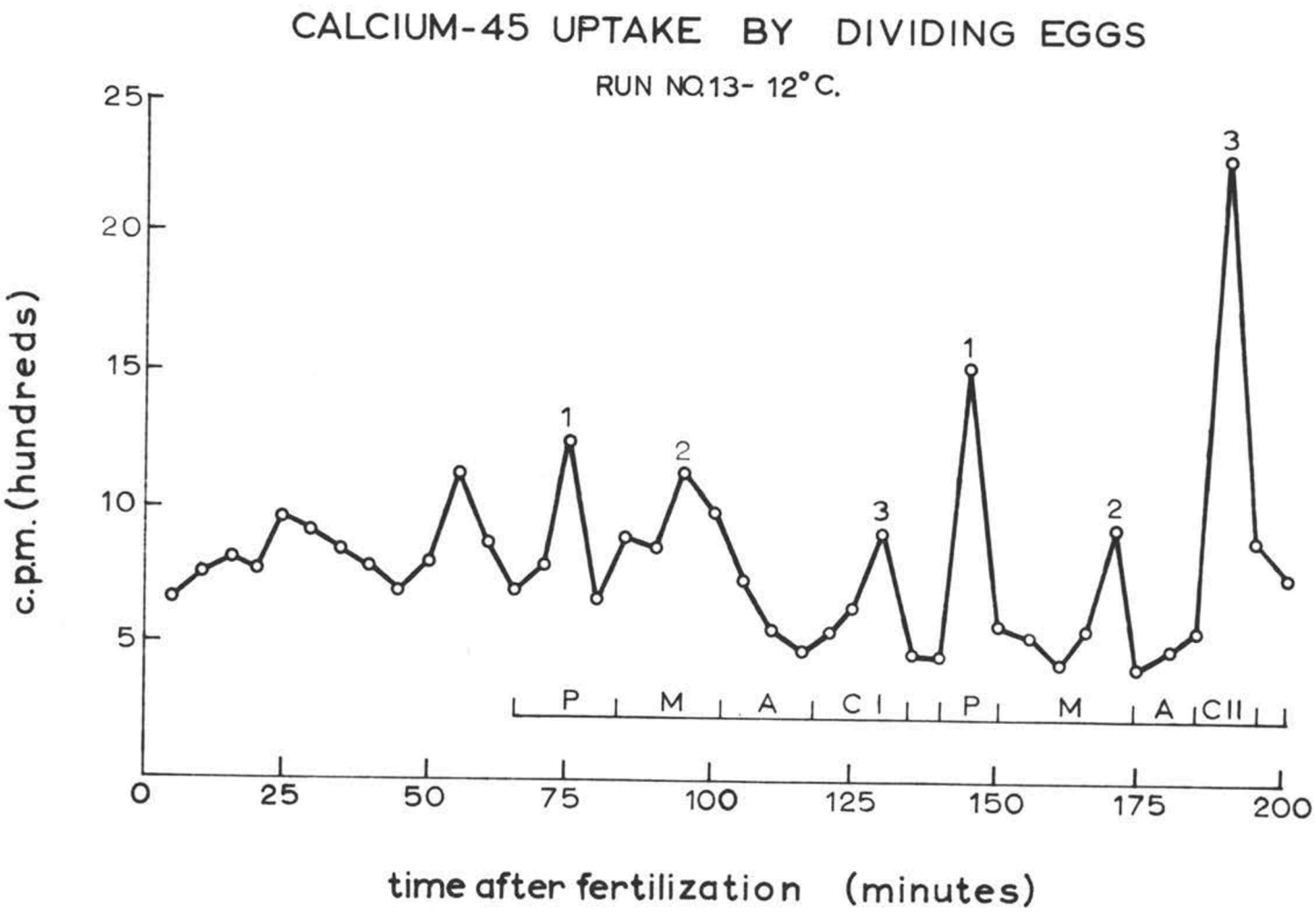
RUN NO. 4 - 15.5°C.
c.p.m. (hundreds)
15
10
5
0
25
50
75
100
125
150
175
200
time after fertilization (minutes)
P
M
A
CI
P
M
A
CII
1
2
3
1
2
3
1
2
3

Fig. 1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RUN NO.13- 12°C.
c.p.m. (hundreds)
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20
15
10
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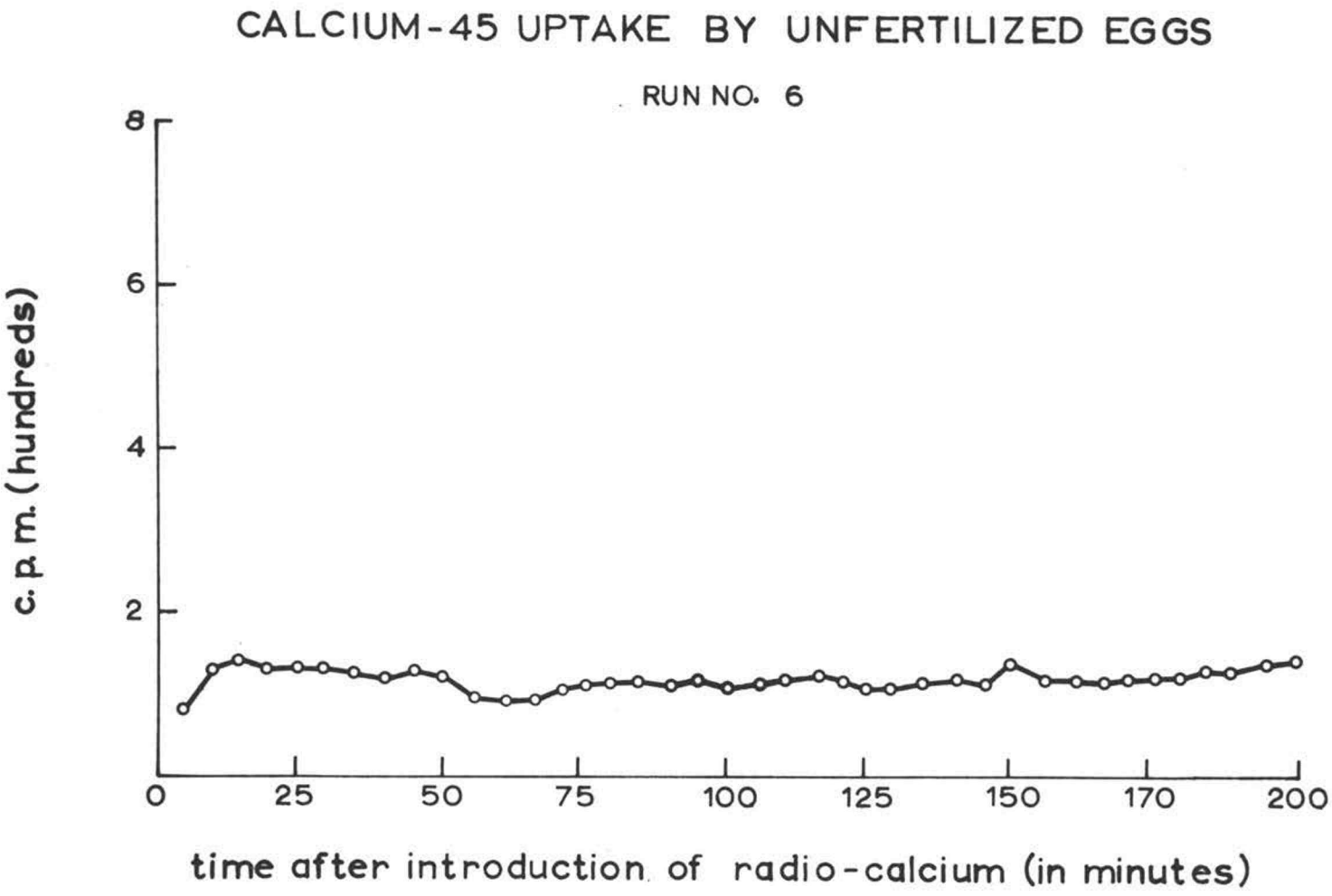
25
50
75
100
125
150
175
200
1
2
3
1
3
2
3
P
M
A
C I
P
M
A
C II
time after fertilization (minutes)

Fig. 2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RUN NO. 6
c. p. m. (hundreds)
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4
2
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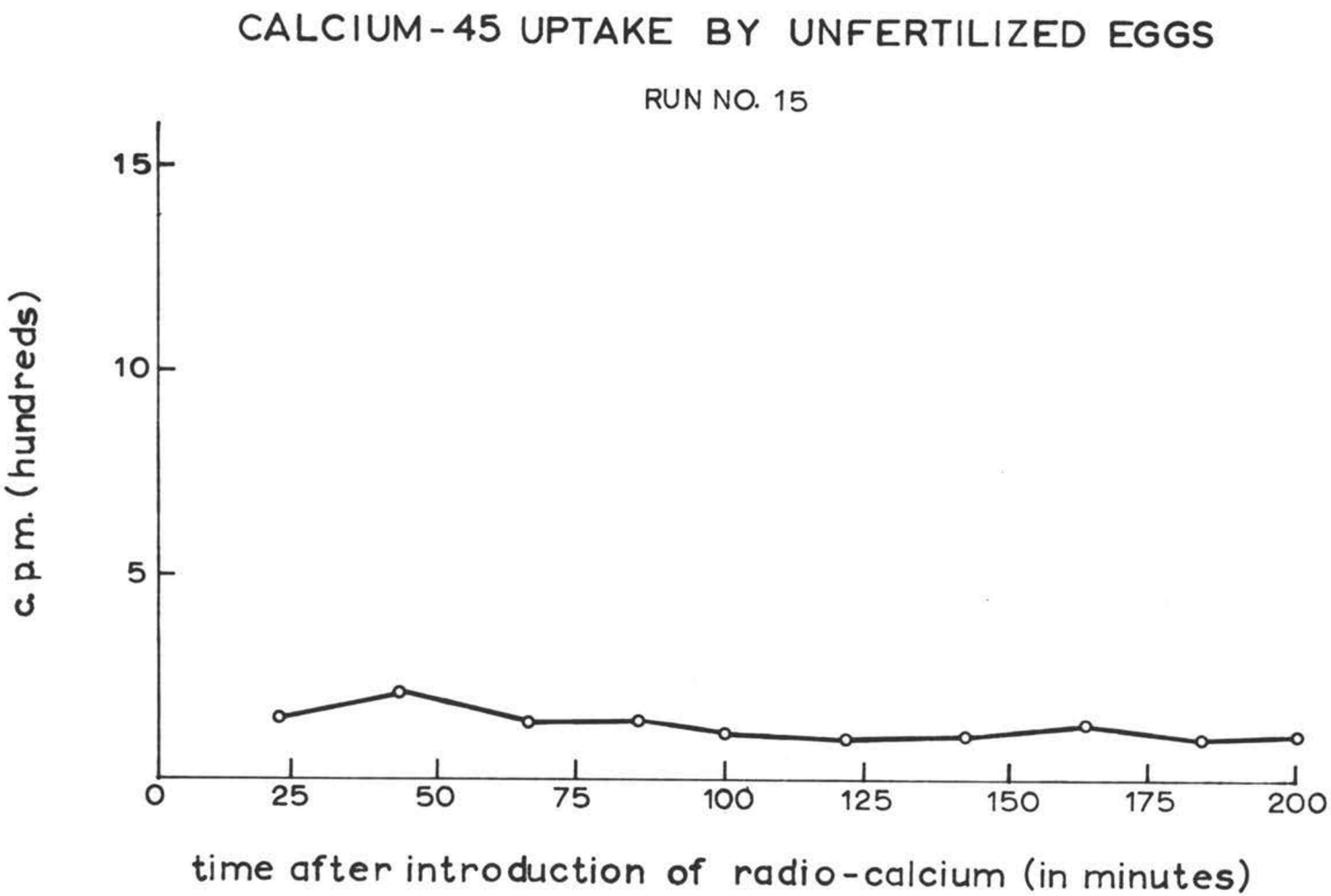
100
125
150
170
200
time after introduction of radio-calcium (in minutes)

Fig. 3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RUN NO. 15
c.p.m. (hundreds)
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5
0
25
50
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100
125
150
175
200
time after introduction of radio-calcium (in minutes)

Fig. 4

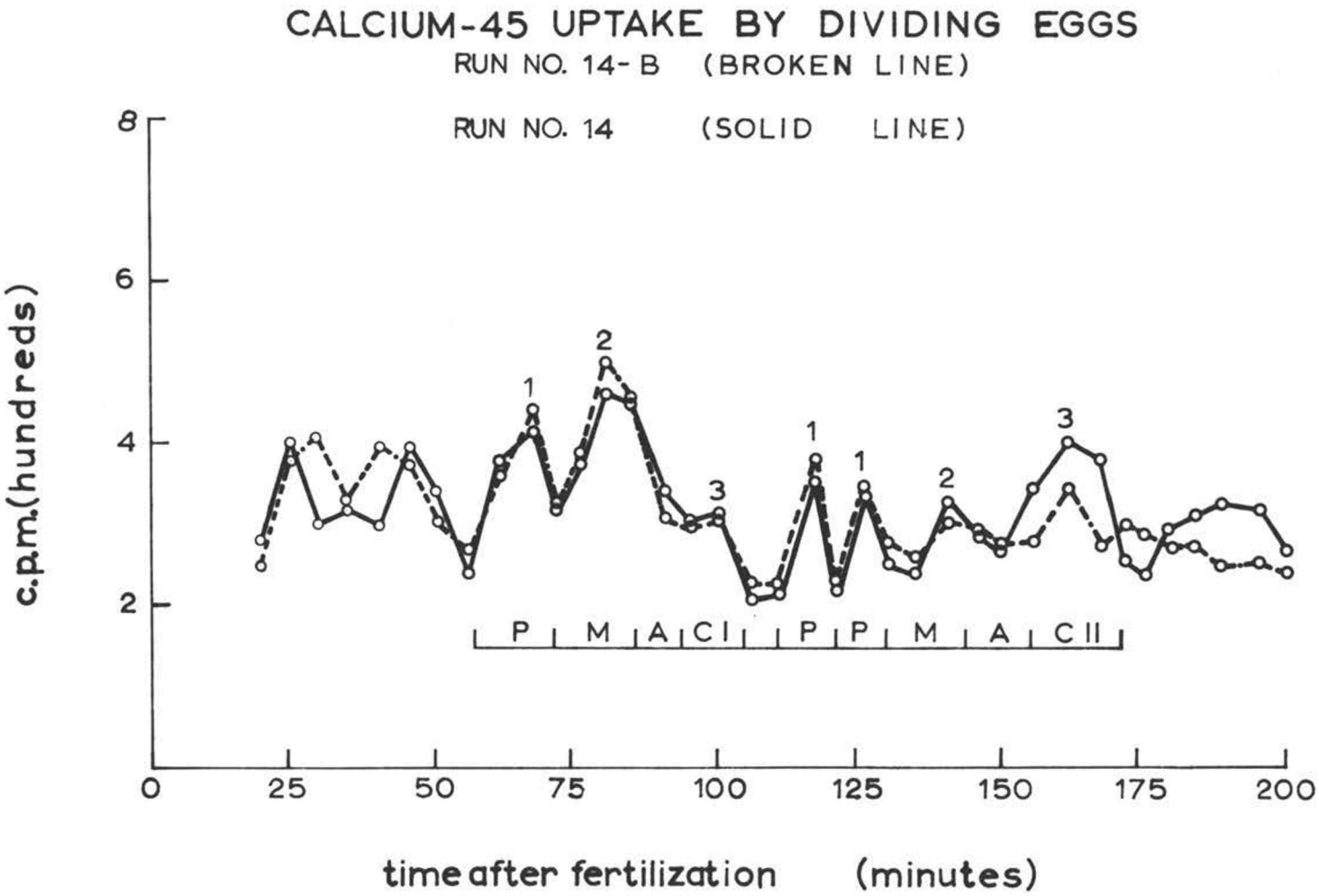
CALCIUM-45 UPTAKE BY DIVIDING EGGS
RUN NO. 14-B (BROKEN LINE)
RUN NO. 14 (SOLID LINE)
c.p.m.(hundreds)
8
6
4
2
0
25
50
75
100
125
150
175
200
time after fertilization (minutes)
1
2
3
1
1
2
3
P M A CI P P M A CII

Fig. 5

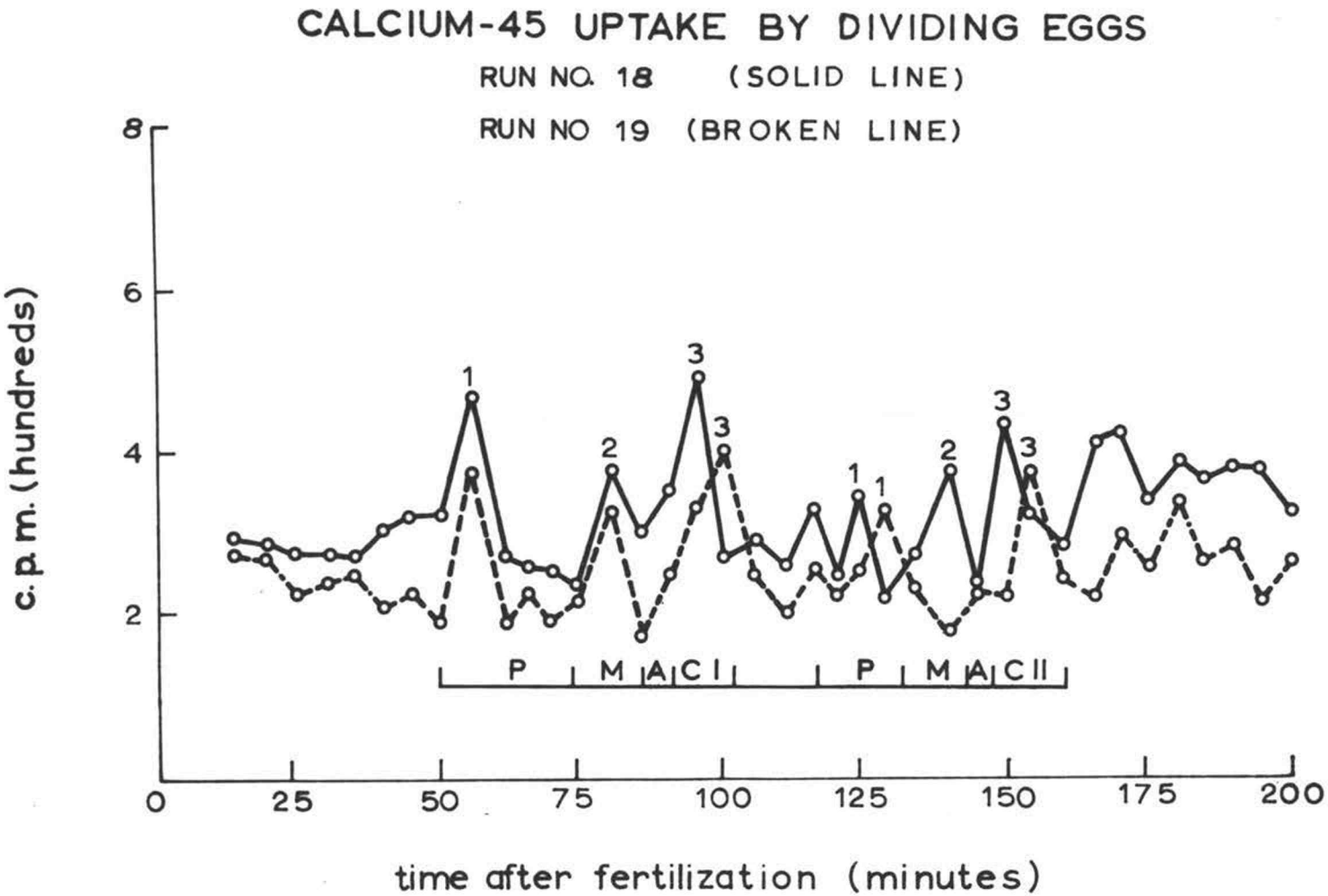
CALCIUM-45 UPTAKE BY DIVIDING EGGS
RUN NO. 18 (SOLID LINE)
RUN NO 19 (BROKEN LINE)
c.p.m. (hundreds)
8
6
4
2
0
25
50
75
100
125
150
175
200
time after fertilization (minutes)
P
M
A
C I
P
M
A
C II
1
2
3

Fig 9

OCCURRENCE OF CALCIUM PEAKS

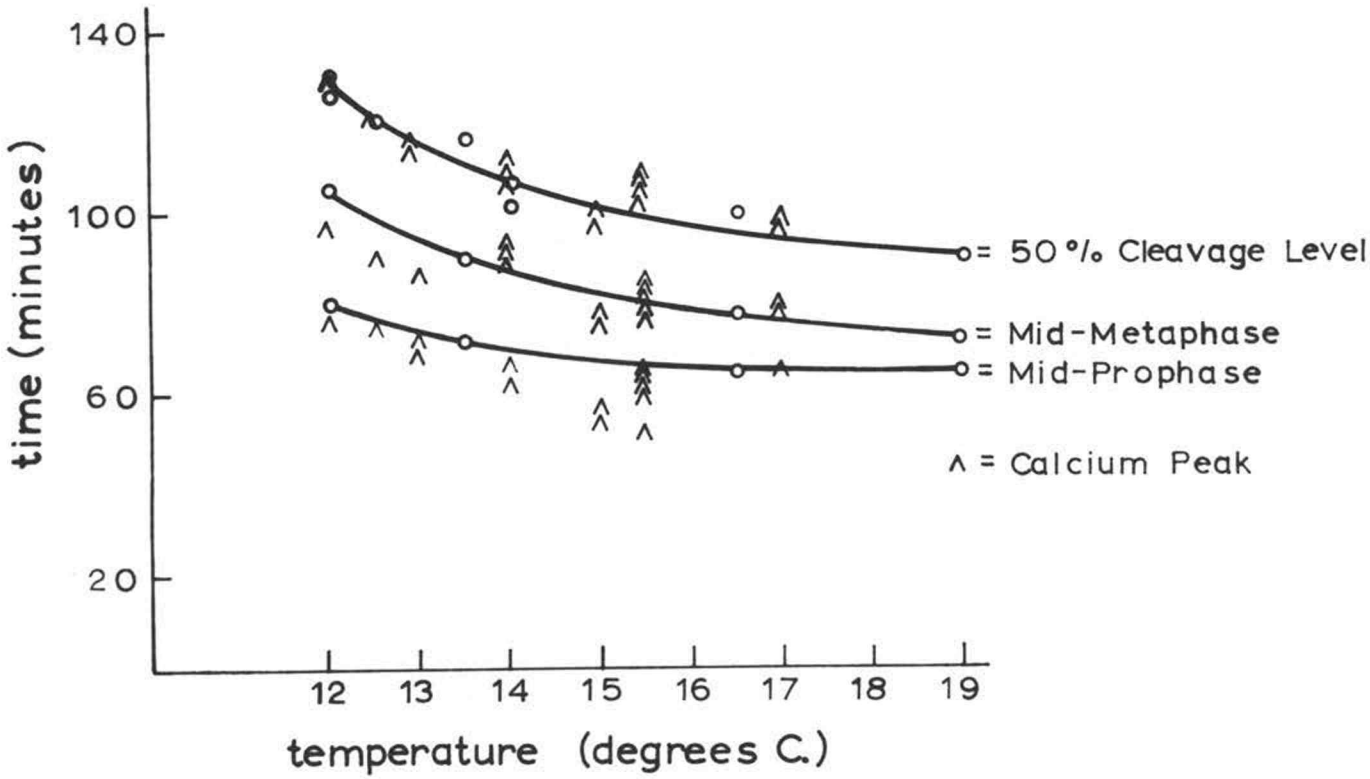

Fig. 7

Fig. 8

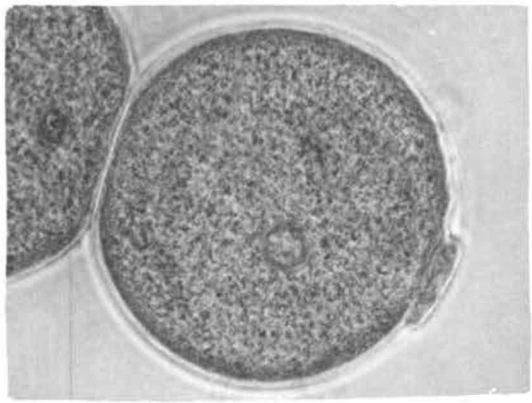

Fig. 8a

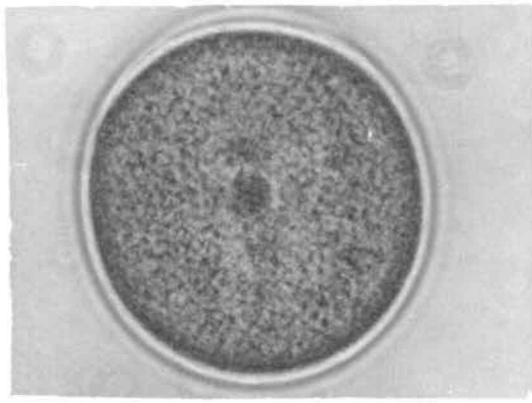

Fig. 8b

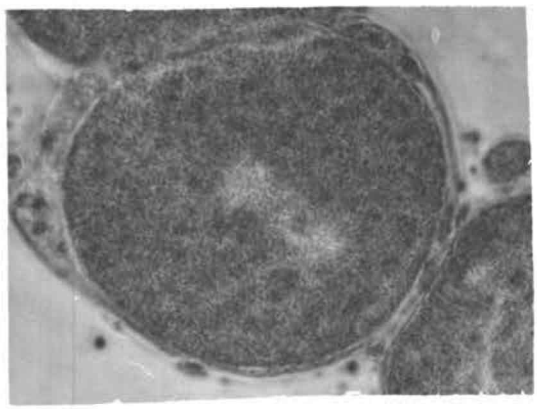

Fig. 8c

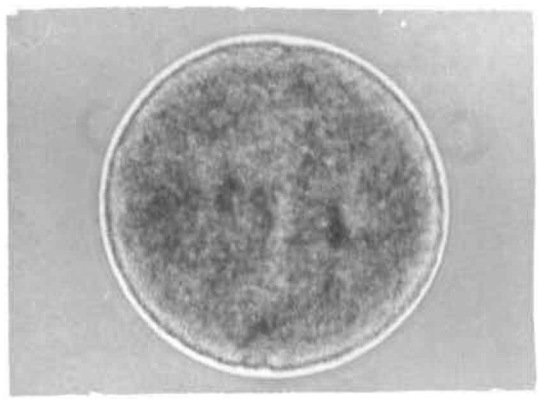

Fig. 8d

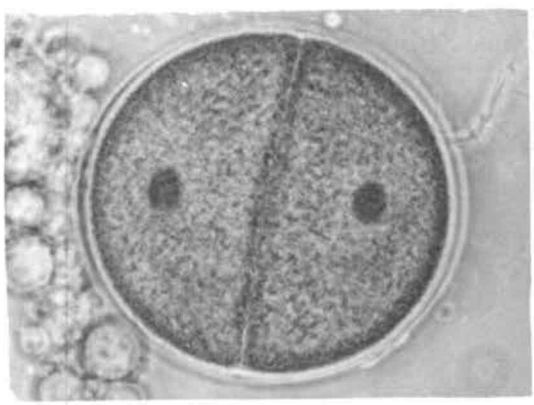

Fig. 8e

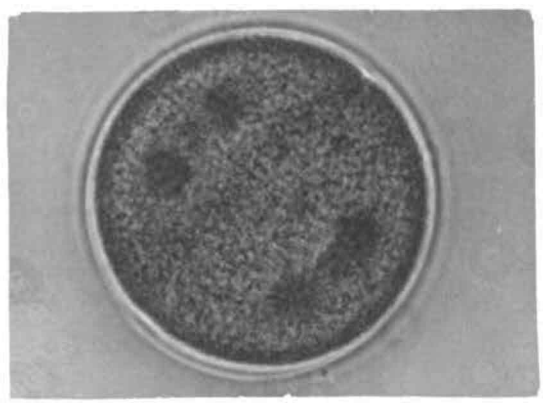

Fig. 8f