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Author
Teng, Mei-Hui

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Mei-hui Teng, James C. Bartholomew, and Mina J. Bissell

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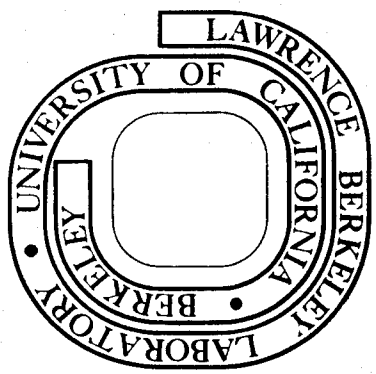

DISCLAIMER

Insulin Effect on Cell Cycle Movement: Analysis of the Kinetics of Growth Parameters in Confluent Chick Cells

Mei-hui Teng, James C. Bartholomew and Mina J. Bissell

Laboratory of Chemical Biodynamics

Lawrence Berkeley Laboratory

University of California

Berkeley, California 94720

Summary

Serum-starved confluent chick embryo fibroblasts can be stimulated to synthesize DNA and undergo mitosis by the addition of insulin at 16 m units/ml medium. Based on the measurement of various growth parameters, the extent of the effect of insulin on growth is comparable to that resulting from the presence of 3% chick serum. However, flow microfluorometry demonstrates that the kinetics of the cell cycle movement in the two cases are different.

Introduction

That hormones regulate the growth of animal cells in cultures has been reported widely (Armelin, Nashikawa, and Sato, 1974; Sibley et al., 1974; for review see Holley, 1975). Insulin is the one hormone that has been much studied in both avian and mammalian systems. The history of the involvement of insulin as a growth-stimulating factor for cells in culture, especially chick cells, is as old as that of tissue culture itself. An early observation on the stimulating action of insulin on the growth of chick cells (Gey and Thalhimer, 1924) prompted investigators to use it in media for organ cultures (Leslie and Paul, 1954) and for permanent cultures of mouse cell lines (Waymouth and Redd, 1965). Temin (1967) has proposed that insulin acts as one of the serum factors that is necessary for the growth of chick embryo fibroblasts. Several peptides with insulin-like activity and growth-stimulating potential for chick cells were then isolated: somatomedin from human plasma (Tell et al., 1973; Smith and Temin, 1975), and multiplication-stimulating factor from calf serum (Pierson and Temin, 1972) and from cultured rat liver cells (Dulak and Temin, 1973a, b).

Other reports suggest either that insulin, by itself, is incapable of triggering the initiation of DNA synthesis, or that it can only stimulate the initiation of DNA synthesis, but not the completion of DNA synthesis and mitosis (Vaheri et al., 1973; Baseman, Paolini and Amos, 1974; Baseman and Hayes, 1975).

Nevertheless, in most cases following the addition of insulin, a substantial increase was reported in the fraction of ^{3}H-thymidine-labeled cells that became acid-precipitable in serum-starved confluent chick cell cultures (Baseman et al., 1974; Baseman and Hayes, 1975). The reason behind the reported lack of concomitant increase in cell number has not been clear. In short, the total picture of insulin action on growth regulation has been confusing and at times contradictory (Temin, 1967; Vaheri et al., 1973; Baseman et al., 1974; Smith and Temin, 1975).

It is possible that the growth-stimulating activity of insulin on chick cells is nonspecific and subject to the particular culture conditions used. On the other hand, the recent demonstration of the existence of insulin receptors on the surface of the chick embryo fibroblasts (Raizada and Perdue, J. Biol. Chem., 1976, in press) indicates that the action of insulin on these cells indeed may be the result of hormone-receptor interaction. In either case, some explanation for these contradictory findings is needed.

Previous studies on the response to insulin have primarily followed growth by measuring ^{3}H-thymidine incorporation into acid-insoluble material, or occasionally by autoradiography, mitotic index, and by cell number. The fate of cells which may have been stimulated to grow by insulin has never been clearly defined. The newly developed technique of flow microfluorometry (Holm and Cram, 1973) for the detection of DNA content per cell makes it possible to monitor cells moving through the cell cycle. To gain more understanding of the possible role of insulin as a growth-stimulating factor, we analyzed in detail the kinetic responses as indicated by various growth parameters, including the cell cycle movement of confluent chick embryo fibroblasts after the addition of insulin. For comparison to insulin we used chicken serum. The results indicate that, in serum-deprived confluent chick cells, the addition of 16 m units insulin/ml of medium alone is capable of initiating DNA synthesis at a rate comparable to the addition of 3% chick serum. The insulin-stimulated cell

population went through DNA synthesis and an eventual mitosis. However, the kinetics of the cell cycle response were distinctly different from those of serum-stimulated cells. In insulin-treated cultures, initiation of DNA synthesis occurred earlier, but the total period for completion of the cell cycle was much longer than for serum-treated cultures. Furthermore, after the addition of insulin, the stimulated cell population moved through the cell cycle from G_1 through S, G_2, M and back to G_1 phase in a synchronous manner. Previous studies from other laboratories have usually dealt with only a few selected time points after insulin addition. The differences observed between insulin and serum-treated cultures can therefore be understood in the light of this present work. Our results are discussed in terms of a model of growth regulation which includes both primary cultures and cell lines.

Results

The inability to grow cells in serum-free conditions has been one of the difficulties in defining an insulin effect. For the present study, serum starvation was carried out at least 12 hr prior to the addition of insulin or serum, as described in the Experimental Procedures.

Results of dose response to insulin shown in Figure 1 indicated that the cell number increased and reached a plateau at a concentration of 16 m units insulin/ml of medium. The increase in cell number was comparable to that reached after 30 hr of treatment with 3% chick serum. Thus, 16 m units insulin/ml and 3% serum were chosen for further comparative studies and are here reported unless otherwise indicated.

Insulin Stability under Culture Conditions

It is important to know the stability of insulin under the experimental conditions used before its effect can be studied. To determine the stability of insulin, a competitive radioimmunoassay for insulin was performed on media from plates with

or without cells and with either 3% chick serum or with 16 m units of insulin/ml. There was no detectable amount of insulin as measured by this assay in the medium containing chick serum. The levels of insulin remaining in the medium originally containing the insulin are shown in Table 1. By 4 hr, 50% of the insulin activity was lost in the culture medium. After 26 hr, there was only 18 to 30% of the insulin detectable in the media.

Cell Cycle Movement following Insulin and Serum Addition

There is considerable evidence that, once resting cells are stimulated by serum for 7 to 10 hr, they are able to traverse the complete cell cycle. Whether or not the same is true for insulin stimulation has been the subject of much argument, as discussed in the Introduction. To investigate the movement of insulin and serum stimulated cells through the cell cycle, we used flow microfluorometry (FMF) (Bartholomew et al., 1976). In this technique, a fluorescent dye that binds quantitatively to DNA is used to measure the distribution of cells in the cell cycle. Cells in G_1 have half the amount of DNA and, therefore, half the amount of fluorescence of cells in G_2+M. Cells in S have DNA contents that range between the value obtained for G_1 and G_2+M cells. The fluorescence emitted from the DNA of each cell was quantified after excitation by a laser beam. The distribution pattern of cells in G_1, S, and G_2+M phases of the cell cycle are demonstrated in Figure 2. If no insulin or serum was included in the medium added to the cells at 0 time, there was no evidence of any stimulation of cells moving out of G_1. In all the control histograms there was a peak of G_2+M cells representing from 5 to 10% of the total population. This peak may be due to cells blocked in G_2+M or may represent two G_1 cells passing through the laser beam simultaneously. Movement of cells out of G_1 was evident in cultures stimulated with insulin or serum. FMF patterns indicated that insulin stimulated cells began entering S before serum stimulated cells. Also, the insulin stimulated cells appeared to move more synchronously through S than the serum stimulated cells.

Although the insulin stimulated cells moved through S faster than the serum stimulated cells, they appeared to move through G_2+M at a slower rate. As seen in Figure 2, the insulin stimulated cells began accumulating in G_2+M starting at about 14 hr; whereas, the serum stimulated cells never really accumulated in this phase of the cell cycle but moved on through mitosis into G_1. This observation is consistent with the increase in cell number occurring in serum stimulated cultures before the increase seen with insulin stimulation, as will be discussed later.

To determine the total number of cells in each culture that had traversed S and G_2 by 22 hr, mitosis was prevented by addition of 10^{-7} M colcemid to replicate plates. The relative amounts of cells in G_2+M indicated in the 22 hr samples of Figure 2 were determined by computer. The total numbers of cells in G_2 in the presence and absence of colcemid are comparable in control cultures. This is also true for insulin-treated cultures. Thus the indication is that only a few cells had gone through mitosis by 22 hr. In serum-treated cultures, however, there were more cells in G_2+M in the presence of colcemid than in its absence (in this experiment 25% versus 9%). A large proportion of cells stimulated by serum had already gone through mitosis by 22 hr. This technique demonstrates the distinct differences in the kinetics of cell cycle movement in insulin and serum-treated cultures. FMF results were compared with the conventional procedures for growth measurement. These were carried out in detail and are discussed below.

Kinetics of ^{3}H-thymidine Incorporation into Acid-Precipitable Fraction

To detect the rate of DNA synthesis, cultures were pulsed with ^{3}H-thymidine for 1 hr at various times following the addition of insulin and serum to serum-deprived cultures. Figure 3 shows the kinetics of ^{3}H-thymidine incorporation into the acid-insoluble fraction. Simply changing the medium caused a slight increase in DNA synthesis in control cultures. Incorporation of ^{3}H-thymidine

increased during 2 to 3 hr after insulin addition and reached a maximal level at 8 to 10 hr. The rate of incorporation decreased thereafter. The onset of response to serum occurred later and the maximal level was lower than the maximal level with insulin.

Measurement of Total Content and Specific Radioactivity of DNA

Figure 3 shows only the rate of ^{3}H-thymidine incorporation into the acid-insoluble fraction. Whether or not this is an accurate measure of DNA synthesis in cultured cells has been questioned at times (Thomas and Lingwood, 1975; Yondale and MacManus, 1975). The total DNA content was therefore measured after the addition of either insulin or serum. Because some cell detachment occurs in the absence of serum, the total DNA level per plate is usually underestimated after insulin addition, especially at early time points. Nevertheless, it can be seen from Figure 4 that an appreciable increase in DNA content has occurred 11 hr after insulin addition. Adding to the problem of underestimating the insulin action, the actual increase in total DNA is initially too small to measure under these conditions. A measurement of the specific radioactivity of DNA after the addition of serum or insulin was therefore undertaken. To ensure measurable radioactivity in DNA, cultures were labeled with ^{3}H-thymidine at 1 μCi/ml for 4 hr. Figure 5 shows the specific radioactivities of the DNA isolated at 5, 11, and 15 hr after growth stimulation. The data agree with that of ^{3}H-thymidine incorporation into acid-precipitable material. A higher rate of incorporation into acid-insoluble material corresponded to the higher specific radioactivity of DNA in insulin-treated culture versus serum-treated culture.

It is possible that, in addition to stimulation of DNA synthesis, insulin also causes an increase in the available radioactive thymidine pool by either increasing the uptake or suppressing the endogenous synthesis of thymidine. This could explain the small discrepancy between the radioactivity in

acid-precipitable material and the specific/radioactivity of DNA on the one hand, and total increase in DNA synthesis on the other hand, especially for early time points.

To bring the various elements of our measurements together and determine their correlation with increase in cell number, the experiment shown in Figure 6 was performed. We confirmed the same picture of cell cycle movement, earlier and synchronous after insulin addition, yet delayed in G_2, and later/unsynchronous after serum addition, yet much faster in G_2. Figure 6a shows a 1 hr pulse of ^{3}H-thymidine incorporation into the acid-insoluble fraction, which, as shown above, correlates with the actual DNA synthesis in our system, as indicated in Figures 4 and 5. While after 27 hr cells in serum continue to synthesize DNA at a lower rate, insulin-treated cultures have gone through one synchronous wave of DNA synthesis. Figure 6b shows the approximate number of cells in S and G_2+M as a function of time calculated from flow microfluorometer studies. Figure 6c shows the percent of labeled nuclei as measured by autoradiography after continuous labeling with ^{3}H-thymidine. Again the data is consistent with the idea that cells in insulin enter S earlier than those that are serum treated, and that the cells show prolonged, but synchronous, S and G_2 phases. The continuous increase in the percentage of labeled nuclei in serum-treated cultures then is due to early division of previously labeled cells. Figure 6d depicts the increase in cell number which is consistent with the above picture. As can be seen in control cultures, despite some DNA synthesis, the number of cells decreases due to their detachment in the absence of serum. Insulin-treated cells remain constant probably due to a steady-state situation created by cell detachment and replacement. They finally go through mitosis after 27 hr. Cells in serum begin to increase in number early and continue to do so during the course of the experiment.

Discussion

Primary or secondary cultures of chick embryo fibroblasts have often been used as models for studying growth regulation. The growth response of these cells to serum and hormones such as insulin has been quantified and compared in various laboratories. The answer to the question of whether or not insulin, like serum, is capable of inducing growth has not been a simple one to find. However, there is no reason to expect that a single compound such as insulin should elicit the same response from the cells as that of a complex mixture like serum. The question then should be "Does insulin indeed elicit a 'growth' response from chick cells and, if so, why such contradictory findings?

There seems to be little agreement in the literature as to the means by which 'growth' or 'mitogenicity' should be measured. The measurement of acid-precipitable radioactivity after a 1 hr pulse with ^{3}H-thymidine has been the most popular procedure for growth measurement. However, because this procedure is dependent on both the rate of uptake of thymidine and its incorporation into DNA, it can be used only as a qualitative estimate of DNA synthesis. In addition, the sampling time is of critical importance. Short sampling times after insulin addition would tend to exaggerate the degree of stimulation of DNA synthesis; sampling times between 15-30 hours would miss the effect entirely due to the synchronous stimulation of DNA synthesis and delayed mitosis. Measurement of the specific activity of DNA isolated from cells at various times after stimulation gives a more accurate rate of ^{3}H-thymidine incorporation into DNA, but is still subject to the variation in the specific activity of the thymidine pool. Measurement of total DNA per dish suffers from the insensitivity of DNA analysis techniques, and is also subject to variation caused by cell detachment. The cell population stimulated to synthesize DNA can be measured by autoradiography, if the cells

are continuously labeled with ^{3}H-thymidine and mitosis is inhibited. This technique is very sensitive, gives some information about the synchrony of the stimulated population, but may give false positives due to the existence of DNA repair. In addition, the procedure is tedious and would tend to exaggerate the percent increase in labeled nuclei after serum addition because of rapid cell division.

In this report, we chose to compare the results obtained by all these techniques and correlated them with flow microfluorometric analysis.

The picture that emerges of insulin action on confluent cultures of chick fibroblasts is as follows: 16 m units of insulin/ml of culture medium, which is close to physiological insulin level, stimulates 30 to 50% of the cells to enter the S phase of the cell cycle after 2 to 3 hr. The cells move rather synchronously, with a mean S residence of 6 to 8 hr, into G_2 phase of the cell cycle. The cells eventually divide, after a mean G_2 residence of 12 to 16 hr. By any definition of 'mitogenicity', therefore, insulin is mitogenic for a significant proportion of cell population under our culture conditions. It can further be concluded that insulin acts at some portion of the G_1 part of the cell cycle. In addition, the kinetics of the insulin response explains some of the contradictory results reported in the literature (Temin, 1967; Vaheri et al., 1973; Baseman and Hayes, 1975). Our data clearly indicate that the cells stimulated by insulin will eventually divide. Yet it is obvious from Figure 1 that increased levels of insulin above 16 m units/ml cannot increase the proportion of cells that are induced to divide. Whether the division of insulin-stimulated cells is an unavoidable, albeit slow, result of DNA synthesis stimulation or whether factors necessary for completion of G_2 and mitosis are slowly provided by cells themselves is not clear.

When this pattern of insulin action is compared to that of 3% chicken serum, the following differences and similarities are observed: The total cell population

that is stimulated to enter S and G_2 is comparable initially (within 15 hr). However, serum-stimulated cells enter S phase a few hours after insulin-stimulated cells, have a lower mean residence in S and a much lower mean residence in G_2. They therefore divide long before insulin-stimulated cells, and some recycle further. In addition, increasing the serum levels leads to increasing the proportion of cells that can divide.

The possibility that there are two distinct populations in chick embryo fibroblasts, only one of which responds to insulin, cannot be ruled out. However, this is an unlikely possibility since even in the presence of serum (unless unusually high levels are used) the gradient response is present (see below). That is, even though there may be a doubling of the total confluent population as measured by cell numbers, some of the increase is due to secondary and tertiary division, since no more than 80% of the total cell population can be labeled by 50 hr (our unpublished data).

The question also arises as to why insulin does not stimulate DNA synthesis in mammalian cell lines to an appreciable extent. Rudland et al. (1974) showed that insulin alone is incapable of stimulating DNA synthesis in Balb 3T3 cells. They suggested that rather than acting as a growth stimulant, insulin is just a permissive factor for other growth-stimulating agents, e.g. fibroblast growth factor. Similar conclusions were drawn for insulin versus prolactin (Mukherjee, Washburn, and Banerjee , 1973). Our unpublished results on confluent Balb 3T3 cells confirmed that little or no movement of cells out of G_1 occurs after insulin addition. However, it has been demonstrated that the action of insulin on DNA synthesis is not confined to avian systems alone. Insulin clearly induces a significant amount of DNA synthesis in primary organ culture of mammary gland (Turkington, 1968). Therefore, either a fundamental difference between cell lines and primary cultures exists, or some attempt to integrate the two types of cultured cells must be undertaken, since both are used as models for growth regulation.

One obvious difference between the cell lines and primary cultures is the 'tightness' of density-dependent inhibition of growth. While cell lines rarely form multiple layers, except in the presence of very high serum conditions, primary cultures can form multilayers even under low serum conditions (Todaro, Lazar, and Green, 1965; Rubin, 1971). There is a common belief that cells in a primary culture never stop growing, but only extend their G_1 period or change their probability of passage through G_1. This would imply that, given enough time and nutrients, all cells in a densely populated primary culture can divide indefinitely. Our data does not support this concept.

We would therefore like to propose a model which encompasses both primary cultures and cell lines in terms of their response to insulin or other single growth factors. Primary cultures, whether avian or mammalian, being closer to the in vivo state, would respond to a three-dimensional growth control, while tightly density-inhibited cell lines are more strigtly two-dimensional. In other words, the cells at the top layers of primary cultures continue to proliferate slowly while the cells at the bottom layers are analogous to those of cell lines and have more stringent requirements for growth induction. A gradient would therefore exist between the bottom and top layers. The cells in the top layers of primary cultures would divide slowly, even without stimuli at high cell densities, as control cultures in Figures 2, 3, 5 and 6 indicate. A mere change of medium, shaking of the plates, etc., would stimulate a slightly higher proportion of the cells. However, at no time would more than 5 to 10% of the population go through mitosis under these conditions, as shown by experiments with colcemid in Figure 2, and autoradiography (unpublished data). Insulin alone would stimulate only those cells that have not reached complete three-dimensional arrest (about 30 to 50% of the population under our conditions). Other factors (or merely a higher concentration of other factors), which are

present in serum, would be required to "turn on" cell lines or cells at the bottom layer of primary cultures. For these cells, then, insulin alone is not sufficient to induce DNA synthesis.

In contrast to Balb 3T3 cells, where no stimulation of DNA synthesis is observed with insulin, Swiss 3T3 cells show a small, but measurable increase in labeled nuclei from 0.2% to 4% (Holley and Kiernan, 1974; De Asua et al., 1975). Interestingly, we have observed in this laboratory that the growth of Swiss 3T3 cells is less stringently controlled than that of Balb 3T3 cells. In other words, there is a direct relation between the 'tightness' of density control and the magnitude of insulin effect. This possibility is under investigation.

The question of whether insulin can stimulate only one round of DNA synthesis while serum can induce more, cannot be answered from our data. The instability of insulin under the culture conditions, as demonstrated in Table I, should be taken into consideration when its long-term effects are examined. If our hypothesis is correct, fresh insulin should still be capable of stimulating another round of DNA synthesis in those cells which have not reached the three-dimensional growth control. Our preliminary data indicates that this may be the case.

The final question, how insulin exerts its effect on growth of some cells, remains unanswered. The binding of insulin to the plasma membrane of liver and fat cells, and human and chick-embryo fibroblasts, has been demonstrated. Some studies have indicated that the receptor site may be the same for insulin and other growth-stimulating hormones such as somatomatin or multiplication stimulating factor (Hollenberg and Cuatrecasas, 1975). Whether this binding alone is sufficient to trigger DNA synthesis mediated by changes in cyclic nucleotides (Otten, Johnson, and Pastan, 1972; Seifert and Rudland, 1974), synthesis of small RNA species (Baseman et al., 1974), or an increase in uptake of nutrients, remains to be seen. Alternatively, the ability of insulin to stimulate growth may be

due to an entirely different aspect of its structure, such as the proteolytic activity reported to be associated with this hormone (Huang and Cuatrecasas, 1975).

Experimental Procedures

Preparation of Cell Culture

Primary fibroblast cultures were prepared from C/O or C/B 10-day old embryos as described by Rein and Rubin (1968). Secondary cultures were prepared on day 4 and seeded at 1 to 1.1 x 10^6 cells per 35 mm plate (Bissell, Hatie, and Rubin, 1973). The cells were grown for 32 to 36 hr to confluency at a density of 1.7 to 1.9 x 10^6 cells per plate in 2.5 ml Medium 199 containing 2% each tryptose phosphate broth, 2% calf serum each, and 1% chick serum. The medium was then removed and cultures were washed three times with warm saline buffer. The cultures were

incubated further for 12 hr or more in 2 ml serum-free Medium 199, at which time the medium was changed to fresh Medium 199 containing serum or insulin. The times of changing the medium and addition of serum or insulin were staggered at times after serum deprivation so that growth parameters could be measured all at one time. Alternatively, factors were added at 12 hr after serum deprivation and growth parameters were measured at times indicated in the figures thereafter. No significant difference in growth response was observed between the two methods.

Flow Microfluorometry

This procedure has been described in detail by Bartholomew et al. (1976). Cultures were washed twice with saline and trypsinized off the plates. Trypsin was then neutralized with soybean trypsin inhibitor and free DNA was digested by DNAase. The single cells were collected by low-speed centrifugation and fixed in 25% ethanol with 15 mM $MgCl_2$. Cells were then stained with mithramycin for 10 min, and analyzed for their DNA content in the flow microfluorometric apparatus modified slightly from that described by Holm and Cram (1973). The results were plotted as cell numbers versus DNA content.

Thymidine Incorporation into Acid-Insoluble Fraction

At each time, the medium was aspirated and 1 ml of warm fresh Medium 199 containing ^{3}H-thymidine was added. The specific radioactivity and total radioactivity of ^{3}H-thymidine are indicated in each figure. The cells were incubated at 39°C for 1 hr. Labeled medium was removed and cultures were quickly washed three times with cold Hank's buffer. One ml of ice-cold 5% trichloroacetic acid (TCA) was then added. After 30 minutes, the TCA-soluble fraction was collected and the precipitates of the cultures were washed twice with cold Hank's buffer. The precipitated proteins were dissolved in 2 ml of 1% NaOH or 0.1% lauryl sulfate (SDS) for 2 hr. Identical samples of 0.4 ml were taken for the

measurements of protein and radioactivity. The TCA-soluble fractions were also measured for their radioactivity. The radioactivity was measured by using 10 ml of scintillation fluid containing 40 ml acetic acid and 80 ml water in 4 liters of PCS (a phase combining system for liquid scintillation counting of radioactive samples from Amersham/Searle). Samples were counted in a Tricarb Packard Scintillation counter.

Autoradiography

Serum-starved cultures received 1 μCi/ml ^{3}H-thymidine in conjunction with the fresh Medium 199 (alone or with insulin or chick serum). At 4, 8, 12, 16, and 27 hr later, cells were trypsinized off the plates, swollen in hypotonic saline, and fixed with a solution of methanol/acetic acid (3:1, v/v). A drop of cell suspension was placed on a clean glass slide and processed for autoradiography as described by Martin et al. (1971).

Isolation of DNA

DNA from culture cells was isolated and measured according to Morimoto, Ferchmin, and Bennett (1975), except that the cells were prelabeled for 4 hr with ^{3}H-thymidine at 2 μCi/ml, 20 Ci/mmole. Quadruple samples were pooled. After hydrolysis in 1 ml of 1 N perchloric acid, 0.4 ml samples were taken for measurement of radioactivity and optical absorbance at 266 nm. Calf thymus DNA was used as standard.

Cell Number Determination

Cultures were washed twice with saline solution and trypsinized off the plates using 2 ml of trypsin at 39°C for 15 minutes. Samples of 0.2 ml were then counted in a Coulter Counter.

Measurement of Insulin Activity

Insulin content was measured by its antibody binding activity by using a competitive immunoassay kit from Schwarz/Mann, Becton Dickinson and Co.

Protein Concentrations

Protein concentrations were determined by the method of Lowry et al. (1951); bovine serum albumin was used as the standard. Auto Analyzer II System (Technicon) was used for the assays.

Media and Reagents

Experimental Materials

Eggs of C/O or C/B type subgroup (Vogt and Ishizaki, 1965) were obtained from H & N, Inc., Redmond, Wash.; culture plates were from Becton, Dickinson and Co., Oxnard, Calif., and Corning Glass Works, Corning, N.Y.

Medium 199 was purchased from Grand Island Biological Co., San Francisco, Calif.; calf serum and chick serum from Microbiological Associates, Bethesda, Md.; bovine pancreatic insulin, colcemid, and calf thymus DNA from CalBiochem, San Diego, Calif.; tryptose phosphate broth from Difco Lab., Detroit, Mich.; Mithramycin from Nathan Belcher of Pfizer Chemical Co., Groton Connecticut; and ^{3}H-thymidine, 20 Ci/mmole from New England Nuclear, Boston, Mass.

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Table 1. Stability of Insulin in the Medium With or Without Cells

Time	Detectable Insulin in the Media	
	Without Cells	With Cells
(hr)	(m units/ml)	
0	16.8 ± 1.5	16.8 ± 1.5
4	9.0 ± 7.8	8.3 ± 4.8
26	4.4 ± 1.4	3.5 ± 0.1
52	3.1 ± 3.6	6.1 ± 1.3

Cultures were prepared as described in Experimental Procedures. After serum starvation, 2 ml of Medium 199 containing 16 m units of insulin/ml were added. The same medium was added to empty plates without cells for parallel experiments. Medium was collected and centrifuged for removing cell debris at indicated times thereafter. Insulin content was measured by competitive radioimmunoassay. Data are presented as the average value of four samples ± standard deviation.

Figure Legends

Figure 1. Dose Response of Cell Growth to Serum and Insulin.

Secondary chick fibroblasts were prepared by procedures described in the Experimental Procedures. A concentrated insulin solution, 25 units/ml, was prepared in Hank's saline without glucose and diluted to 0.65 units/ml with Hank's saline before use. After serum starvation, various volumes of this insulin solution and serum (taken directly from commercial bottle) were added to cultures in fresh Medium 199. Thirty hours later cells were trypsinized and counted in a Coulter Counter. The cell numbers obtained from cultures with added insulin or serum were divided by that from the control culture, and expressed as a percentage increase for insulin (—●—●—●—) or serum (—o—o—o—o—).

Figure 2. Cell Cycle Movement After Insulin or Serum Addition.

The cell population at the times indicated were harvested and analyzed by FMF as described in Experimental Procedures. The results are presented as cell number vs. DNA content (arbitrary units) for each time point. a) Serum-starved cultures were provided with fresh Medium 199 with or without insulin or chick serum. Cells were harvested at indicated times. b) Colcemid added at concentration of 10^{-7} M to parallel set of plates at 3 hr after medium change. Cells were harvested at 22 hr and processed for flow microfluorometry.

The numbers in parentheses indicate the total cells in G_2 + M in each case.

Figure 3. Insulin and Serum Effect on the Kinetics of Thymidine Incorporation into Acid-Precipitable Fraction.

Preparation of secondary chick cell cultures and measurement of ^{3}H-thymidine incorporation were described in the Experimental Procedures. After serum starvation, insulin and serum were added at concentrations of 16 m units/ml and 3%, respectively, at various times. Thymidine incorporation for all samples was carried out at once by labeling cultures for 1 hr with 1 ml of Medium 199 containing ^{3}H-thymidine, 2 μCi/ml and 20 Ci/mmole. The result is expressed as dpm per μg protein: insulin (-●-●-●-), serum (-o-o-o-), control without medium change (--Δ--Δ--Δ--), control with medium change (-▲-▲-▲-).

Figure 4. Kinetics of Increase in Total DNA in the Presence of Insulin or Serum.

After serum starvation, fresh Medium 199 either alone or with insulin or serum was applied to all cultures. After various time intervals, ^{3}H-thymidine was added at a concentration of 1 μCi/ml and 10 Ci/mmole, and incubated for 4 hr before cell harvest. Isolation of DNA and measurement of specific radioactivity were described in Experimental Procedures and in that of Morimoto et al., 1974. DNA content per dish represents the amount isolated from cells harvested at times indicated. Values are averaged from quadruple samples; insulin (-●-●-●-), 3% chick serum (-o-o-o-).

Figure 5. Specific Radioactivity of Isolated DNA after the Addition of Insulin and Serum.

Experimental Procedures were as described in Figure 4. The specific radioactivities of DNA in cultures labeled from a) 1.5 to 5.5 hr ▨ ; b) 7 to 11 hr ▩ ; c) 11 to 15 hr ▥ are depicted.

Figure 6. The Kinetics of Four Growth Parameters after the Addition of Insulin or Serum.

The techniques for ^{3}H-thymidine incorporation, Figure 6a; flow microfluorometric analysis, Figure 6b; autoradiography, Figure 6c; and for cell counting, Figure 6d; were described in Experimental Procedures. The ^{3}H-thymidine added was at 1 μCi/ml and 20 Ci/mmole for incorporation into acid insoluble fraction, Figure 6a; and 1 μCi/ml, with reduced specific activity, 5 Ci/mmole, for autoradiography, Figure 6c. The proportions of cells at S and G_2 + M phases shown in Figure 6b were obtained by integration of flow microfluorometric patterns: Fresh medium (-▲-▲-▲-), insulin (-●-●-●-), and serum (-o-o-o-).

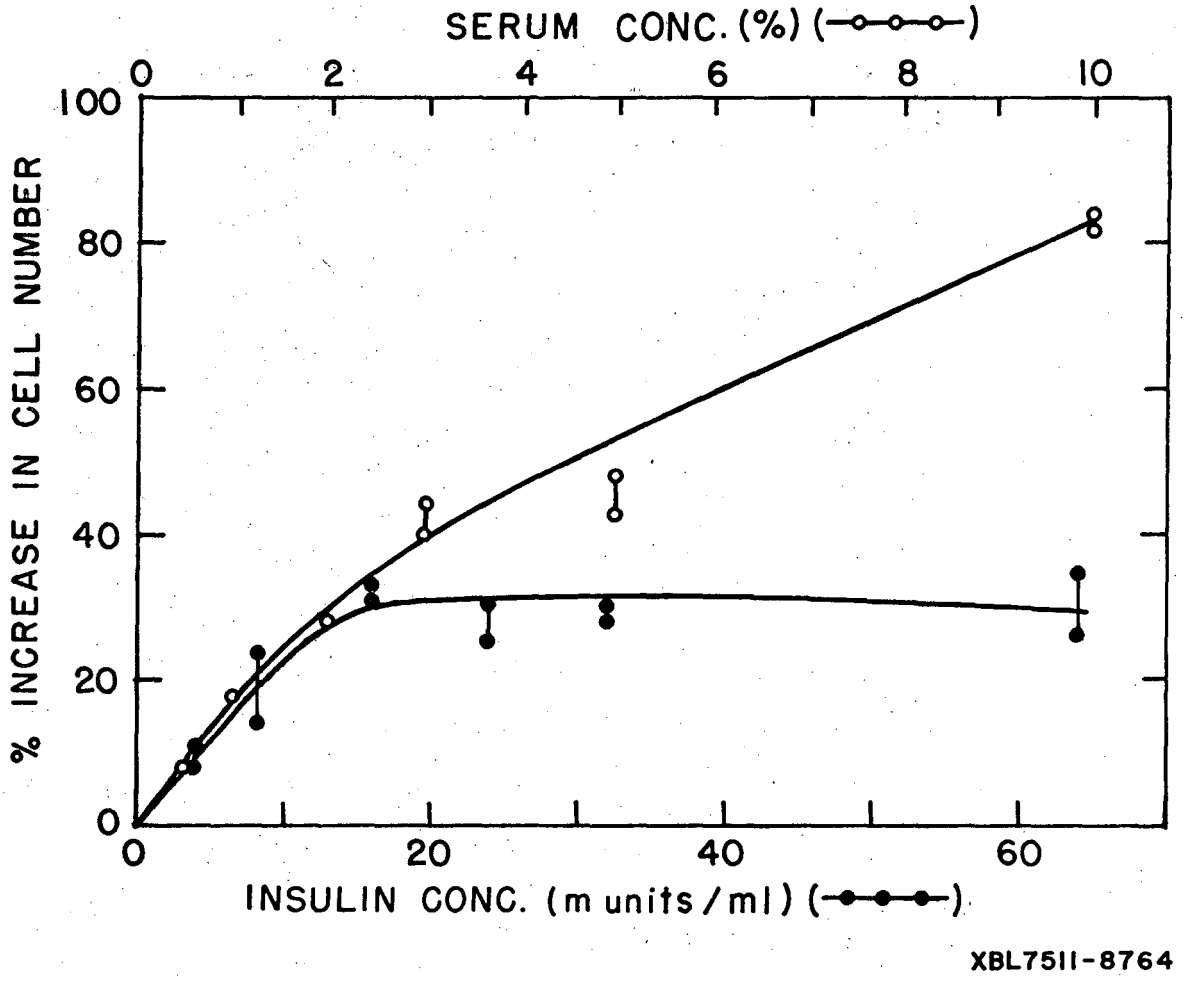

Figure 1
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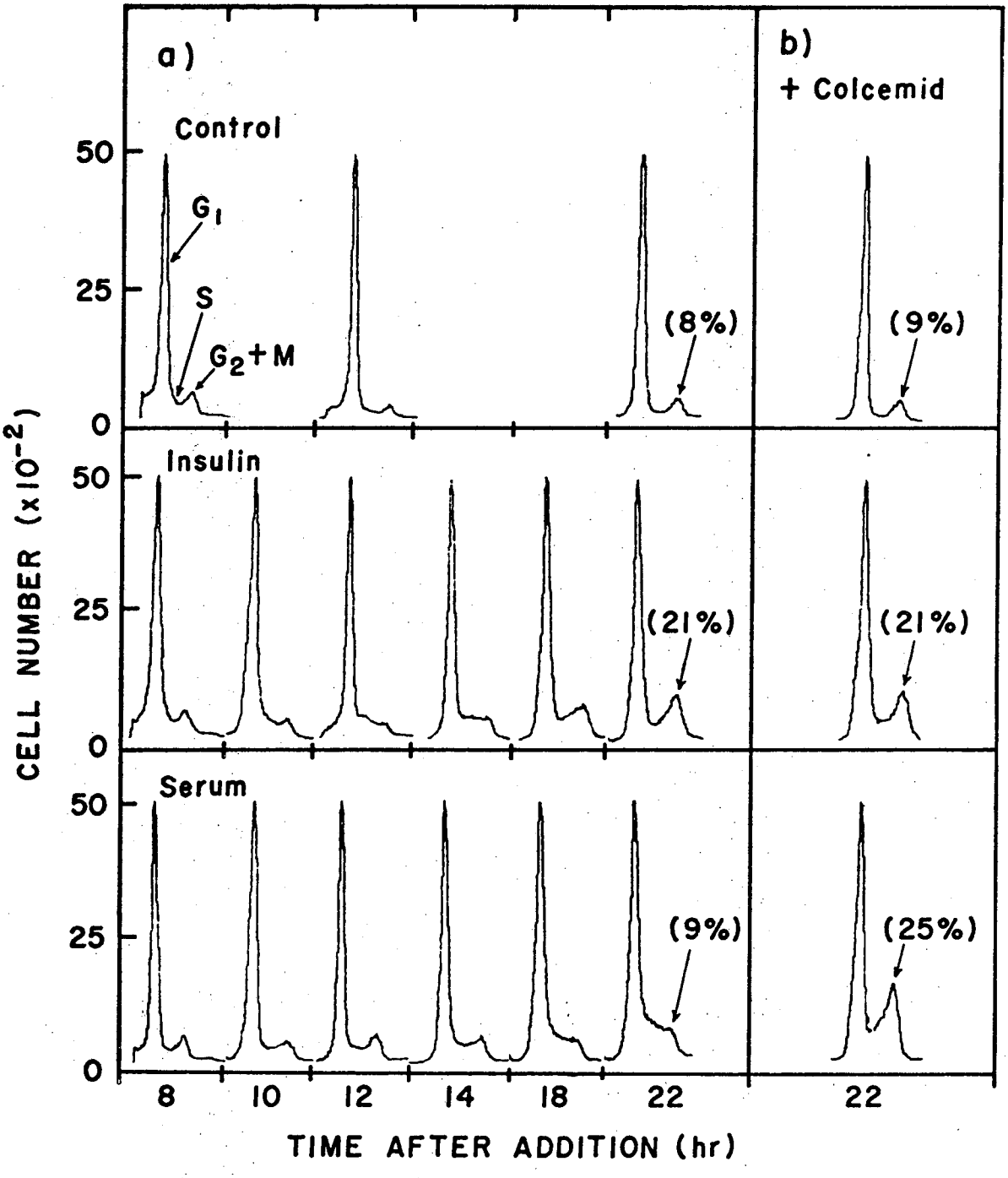

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Figure 2
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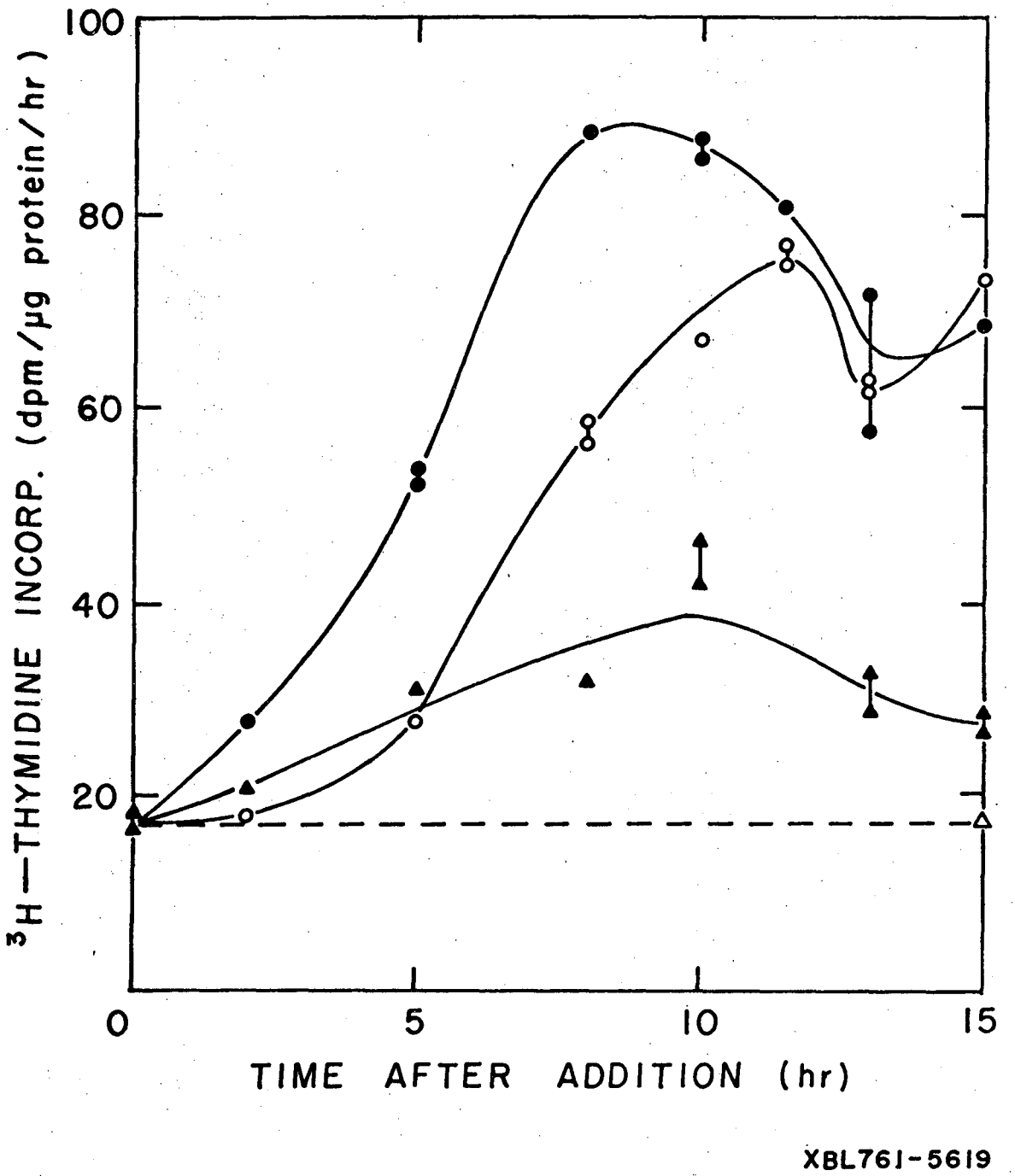

Figure 3
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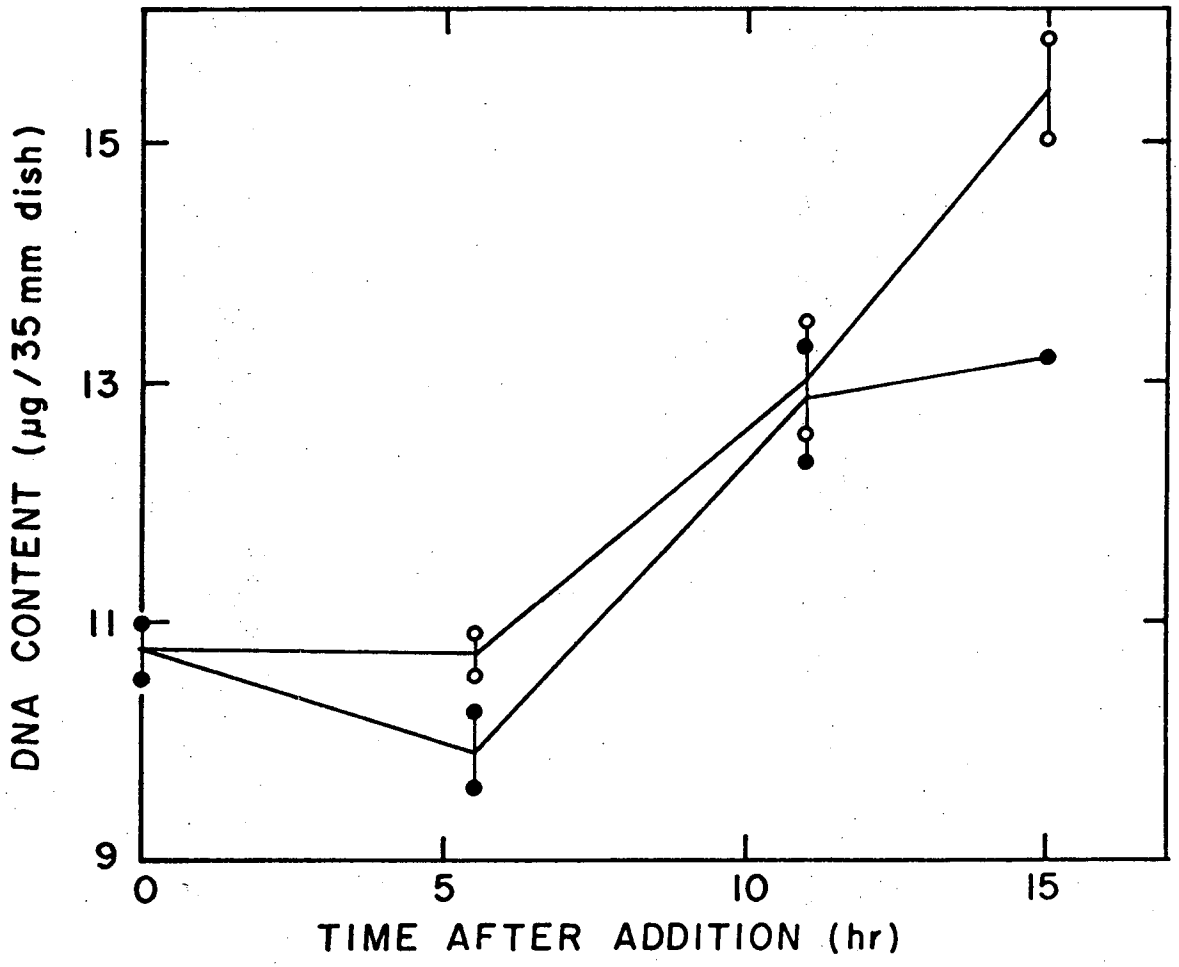

Figure 4
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TECHNICAL INFORMATION DIVISION
LAWRENCE BERKELEY LABORATORY
UNIVERSITY OF CALIFORNIA
BERKELEY, CALIFORNIA 94720