NON-IDENTICAL MECHANISMS OF MITOTIC ARREST BY RESPIRATORY INHIBITORS IN PEA ROOT TIPS AND SEA URCHIN EGGS

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ABSTRACT

The adenosine triphosphate (ATP) content of pea root tips is about 0.40 mmole/kg fresh weight. The effects of partial and complete anaerobiosis, and of carbon monoxide and hydrogen cyanide, on the ATP level are described. The ATP content was shown to correspond closely to the oxygen uptake under these conditions. However, there was no relation between the ATP level and the rate of mitosis, a situation which is in contrast with that in sea urchin eggs. In anaerobic conditions, mitoses in pea root tips could continue at a reduced rate, even though the ATP content had fallen to 1.5 per cent of the normal value in air The ATP level in anaerobic conditions corresponded closely to the expected rate of ATP regeneration from known anaerobic sources of energy. Calculations show that even this severely restricted supply of energy would be more than adequate to drive the anaphase chromosome movements, so it is suggested that the concept of a mitotic energy reservoir is superfluous in root tips. No evidence could be found for the involvement during mitosis in sea urchin eggs of a non-respiratory ferrous complex such as occurs in pea root tips. Hence the dilemma remains, that whereas mitoses in both sea urchin eggs and pea root tips are arrested by respiratory inhibitors, yet the biochemical mechanisms of the arrest in the two types of cell are totally distinct.

In a variety of tissues, the progress of dividing cells through the visible phases of mitosis has been shown to be relatively indifferent to inhibition of the energy-yielding metabolic pathways. These observations were interpreted by Bullough (3) to mean that preparations for the oncoming mitosis, including the generation of the necessary energy, were completed before prophase. This idea was elaborated by Swann (4) into the stimulating concept of a hypothetical "energy reservoir," which was supposed to be progressively filled during interphase, and subsequently drained during mitosis.

Although the concept of an energy reservoir was developed by these authors specifically to explain their own observations on cell divisions in mouse ear epidermis (5) and sea urchin eggs (4) respectively, later reviewers have tended to regard the hypothesis as a generalization of more universal validity (6–9). However, there are indications in the older literature that the cells of various other tissues are in fact liable to mitotic arrest by respiratory inhibition applied during visible mitosis. In the past two years, arrest, or at least drastic slowing, of visible mitosis has been conclusively demonstrated in careful studies of three different tissues, by Amoore (10, 11) working with pea root tips, by the late I. A. Utkin (12) on mouse corneal

epithelium, and by Epel (1) on sea urchin eggs. These three recent studies are therefore in complete agreement that mitoses in progress can be arrested at any stage by respiratory inhibitors.

Utkin's (12) work was unfortunately not carried beyond cytological examination of the tissue, but he made the natural suggestion that, although a certain amount of energy might be stored, some additional energy is required during mitosis. Both Amoore and Epel conducted physiological and biochemical studies of the tissue, aimed at discovering how much energy might actually be available to the tissue under conditions of metabolic inhibition. Amoore (11) measured oxygen uptake by the pea root tips and Epel (1) measured adenosine triphosphate (ATP) content of the sea urchin eggs. In this way, both authors were able to compare (or contrast) the observed rates of mitosis with the probable energy levels existing in the tissue, and hence to develop hypotheses as to the biochemical mechanisms by which the respiratory inhibitors brought about mitotic arrest.

Rather unexpectedly, the conclusions respecting sea urchin eggs appear to be completely different from, and even opposed to, the situation believed to exist in pea root tips. Thus Amoore (13) could find no correlation between rate of mitosis and rate of respiration in pea root tips. Rather, the arrest of mitosis appeared to be brought about by interference with a non-respiratory ferrous complex which is essential to the mitotic process (2). Furthermore, mitoses continued while the calculated energy production was reduced by oxygen lack to only 1 per cent of normal, in keeping with the energy reservoir hypothesis (11).

In contrast, Epel (1) has found a very close correlation between rate of mitosis and ATP content of sea urchin eggs, suggesting that the arrest of mitosis was caused by the fall in ATP level. There is no indication in his data of any additional involvement of a non-respiratory ferrous complex. Finally, mitoses came to a halt when the ATP level was still as high as 50 per cent of normal, rendering an energy reservoir hypothesis unnecessary.

It is disconcerting to find so fundamental a disagreement between the postulated mechanisms by which respiratory inhibitors bring about mitotic arrest in two different tissues. At the cellular level, a greater degree of similarity in the details of so basic a physiological function as mitosis could have been anticipated. Accordingly it was considered mandatory to continue the study of this contrast, by performing the complementary experiments to those described above. That is, Epel's method of ATP analysis would be applied to pea root tips, and Amoore's procedure for diagnosing the mitotic ferrous complex would be tried on sea urchin eggs. It may be stated at the outset that the new results obtained have been found to confirm and amplify the existing information, without resolving the basically conflicting situations in pea roots and sea urchin eggs.

METHODS

Root tips (2.0 mm) were cut from 2-day seedlings of peas (*Pisum sativum*, var. Alaska). The general experimental methods have been described in the earlier papers. Root tips were incubated at 25°C in the dark.

ATP was estimated by the firefly luminescence method of Strehler and Totter (14), as modified by Epel (in preparation) along lines similar to those of Wahl and Kozloff (15). At the end of the incubation the root tips (usually 10) were immediately plunged into a test tube containing 5 ml of 0.05 M aminotrishydroxymethylmethane/sodium maleate buffer, pH 7.0, which had been preheated in a boiling water bath. After 10 minutes at 100°C, the tubes were transferred to crushed ice, and finally placed in the deep freeze overnight. After thawing, the supernatant fluid was decanted with rinsings and made up to 10.0 ml. Samples (0.70 ml) of this extract were taken for the ATP estimations by Epel's procedure. Tests showed that ATP added to the boiling root tips, or to the final extract, was almost quantitatively recovered (95 per cent). The reliability of this method of extracting ATP with boiling buffer was also checked by an alternative extraction with ice cold perchloric acid, which gave concordant results.

Respiratory measurements were made at 25°C by standard Warburg manometric techniques (16), using 50 root tips per flask. Aerobic oxygen uptake was measured for 4 hours in air. Anaerobic carbon dioxide output was measured in an atmosphere of 95 per cent N_2 and 5 per cent CO_2, with 3.0 ml of 2.25×10^{-3} M $NaHCO_3$ (final pH 6.5) in the main well. The apparent CO_2 evolution by the root tips was corrected for CO_2 displaced by acid production (if any). Acid production was estimated by tipping 0.3 ml of 3 N H_2SO_4 from the side arm after the initial equilibration, and (in further manometers) after 1 hour and 4 hours. Any decrement in the resulting CO_2 evolution represented acid production. When the CO_2 evolution had ceased, the Warburg flasks were deep frozen overnight, then thawed, and the supernatant fluid was transferred with rinsings and made up to 9.0 ml. Samples (4.0 ml) were taken for orthophosphate estimation by the method of Allen (17) scaled down to a final volume of 5.0 ml.

Sea urchin eggs (*Strongylocentrotus purpuratus*) were obtained by standard procedures (18). After fertilization they were incubated in stoppered 125-ml conical flasks at 15°C, either in darkness or in the light (6 inches from a 100-w filament lamp; light intensity at the flasks about 800 foot-candles). At intervals samples of eggs were removed, fixed with acetic alcohol, examined by phase contrast microscopy, and assigned to the various phases of mitosis. The average development of a given sample of 100 eggs were expressed as their weighted mean development time, in minutes. The ratio of the observed development time, after applying the inhibitor, to the actual time of exposure to inhibitor, expressed as a percentage, gave the relative rate of progress of the cells through mitosis. An example of the application of this procedure has been given by Epel (1).

RESULTS

Effect of Respiratory Inhibition on ATP Content of Pea Root Tips

This series of experiments deals with the application of Epel's procedure for ATP analysis to root tips, to search for a possible correspondence between ATP level and rate of mitosis, such as has been found in sea urchin eggs (1).

The absolute ATP content of freshly cut pea root tips (sampled within 3 minutes of excision) was found to be 2.35 mμmole/mg dry weight. The percentage dry matter of the root tips was 17 per cent. Expressed on a fresh tissue basis, this is equivalent to 0.40 mmole ATP/kg fresh weight.

Existing knowledge of the ATP content of plant tissues is rather scarce. However, it is interesting to find that the above analyses on pea root tips agree quite closely with the few available values in the literature for seedlings of the same age, even though the species and the methods of ATP estimation were different. Thus, from data obtained by Albaum (19) with muscle adenylic deaminase on the ATP phosphorus of whole, 2-day *Phaseolus aureus* seedlings, it is possible to calculate that they contained 0.47 mmole ATP/kg fresh weight. Šebesta and Šorm (20), using a viscometric method, found that decotyledonized 2-day *Phaseolus vulgaris* seedlings contained 0.45 mmole ATP/kg fresh weight. Finally, Cherry and Hageman (21), who separated the nucleotides by column chromatography and then estimated them by ultraviolet absorption, reported that the scutellum and embryonic axis of 2-day *Zea mays* seedlings contained 1.8 mμmole ATP/mg dry weight. Šebesta and Šorm (20) pointed out that, as a rough generalization, the average ATP concentration in plant tissues is only about one-tenth of that in animal tissues.

The ATP content of pea root tips incubated in air for up to 4 hours proved to be very sensitive to the incubation conditions (Fig. 1). If the seedlings were not rinsed, and the root tips were kept in a moist atmosphere in a Petri dish, but out of contact with liquid water, then the ATP content decreased only slowly during 4 hours. However,

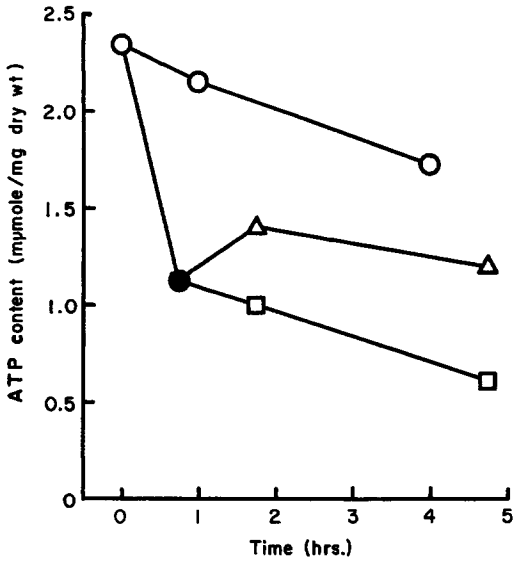

FIGURE 1 Dependence of ATP content of excised root tips on incubation conditions. Air, 25°C. Open circles, in Petri dish, no water; triangles, on filter paper in specimen tube, wet; squares, in Thunberg tube, wet. Solid circle, see text.

most of the experiments have called for the cutting of a large number of root tips, which were accumulated in a shallow layer of water (about 1 mm deep) in a Petri dish. By the time enough tips had been amassed (about 45 minutes), the ATP content had fallen to about half the original value (solid circle in Fig. 1). The subsequent incubation was performed either in stoppered specimen tubes or in Thunberg tubes. The specimen tubes, 1 inch in diameter, contained 0.4 ml of water and two circles of filter paper, on which the root tips were placed. The Thunberg tubes contained 0.01 ml of water, in which the root tips were rather crowded. Some initial recovery of ATP content was ob-

served in the specimen tubes, but the earlier fall in ATP content continued in the Thunberg tubes. It is probable that free access of air to the root tips was somewhat restricted by the presence of static water, more seriously so in the Thunberg tubes. This dependence of ATP level upon the nature of the incubation vessel is in contrast with the indifference of the rate of mitosis to the various experimental vessels employed in the earlier work (13). Accordingly, the ATP levels resulting from application of respiratory inhibitors were always

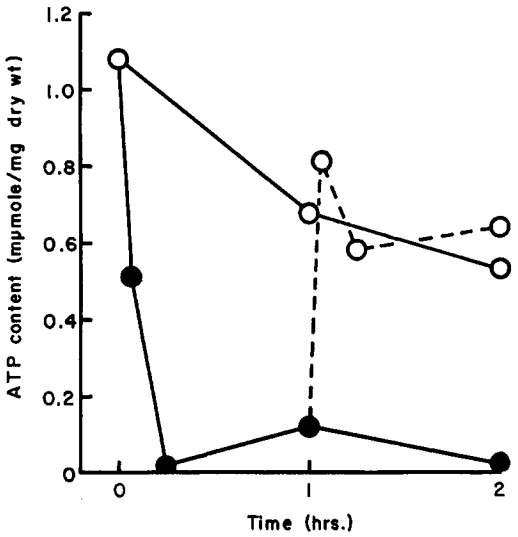

FIGURE 2 Early changes in ATP content of root tips on imposition and relief of anaerobic conditions. Thunberg tubes. Open circles, air; solid circles, nitrogen. Broken line shows effect of admitting air to anaerobic tubes after 1 hour.

compared with the level observed in similar control vessels without inhibitor.

The ATP content decreased rapidly when the root tips were placed in a nitrogen atmosphere, and recovered even more rapidly when air was readmitted (Fig. 2). After 4 minutes of anaerobiosis the ATP level had fallen to half the initial value, and it was down to about 2 per cent by 15 minutes. Restoration of the ATP level was complete in less than 4 minutes after the nitrogen was replaced with air. This demonstrates the necessity for fixing the root tips in boiling buffer as soon as possible after opening the Thunberg tubes. In practice this was accomplished in less than 20 seconds. A small (10 per cent) increase in ATP took place at about 1 hour after the root tips were placed in nitrogen. Even higher levels (up to over 40 per cent) have been observed in other experiments, suggesting that after some delay an anaerobic mechanism for ATP generation is brought into effect. However, after 2 hours in nitrogen this source of ATP was also exhausted.

It may be mentioned that the control ATP levels observed on incubating root tips in Thunberg tubes in air were the same, whether natural air was present, or air "reconstituted" from the appropriate mixture of commercial high purity nitrogen and oxygen. This test was conducted in view of the assertion by Bullough and Laurence (22) that so-called pure nitrogen, and even pure oxygen, from commercial sources may contain an unidentified toxic ingredient capable of causing necrosis of mouse ear epidermal cells. Another test showed that roughly anaerobic conditions (0.1 per cent oxygen) and highly anaerobic conditions (<0.001 per cent oxygen) had identical effects on the ATP level, a finding which is in contrast with the quite different effects of these two oxygen tensions on the rate of mitosis (13).

In two respects the above described results on partial and extreme oxygen lack already suggest that there can be little correspondence between ATP content and rate of mitosis. Thus, a mild deficiency of oxygen caused by a thin layer of water had drastic effects on the ATP level (Fig. 1), whereas the rate of mitosis was known to be almost unaffected. Conversely, two different levels of severe oxygen deficiency, resulting in different rates of mitosis, had identical effects on the ATP level. Accordingly, three different ways of bringing about respiratory inhibition were tested for their effects on ATP content. The three inhibitors chosen were oxygen lack, carbon monoxide, and hydrogen cyanide, because they had been extensively studied in the earlier work. The concentrations selected were those which were already known (2) to reduce the relative rate of mitosis in pea root tips to 50 per cent of the normal rate in air. These were 0.33 per cent oxygen (in nitrogen); 40 per cent carbon monoxide (plus 20 per cent oxygen, in nitrogen); and 6.8×10^{-4} M hydrogen cyanide (in air). If the pea roots behaved like sea urchin eggs (1), then the presence of these concentrations of inhibitor, having the same effects on the rate of mitosis, should have the same (or closely comparable) effects on the ATP level.

The results are shown in Fig. 3. It is clear that the effects of these three inhibitors on the ATP levels are perfectly distinct. The carbon monoxide had barely any effect, whereas the oxygen lack eventually decreased the ATP almost to nothing, and the cyanide yielded an intermediate result. The ATP levels at the end of 4 hours of incubation in the presence of inhibitors were compared with the control samples incubated in air for the same

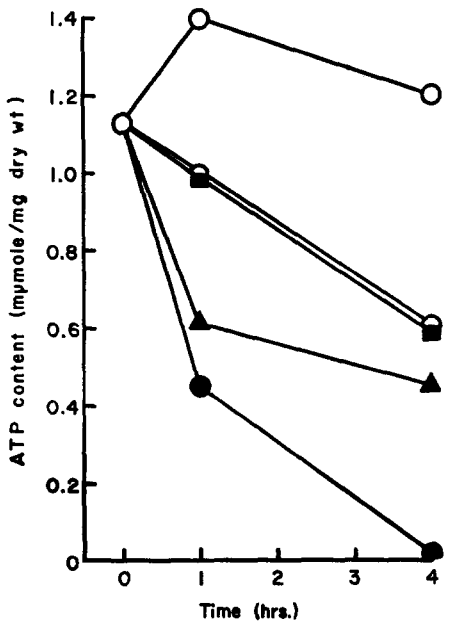

FIGURE 3 Effect of respiratory inhibition on ATP content of root tips. Open circles, air (upper curve, specimen tube; lower curve, Thunberg tube); solid squares, 40 per cent carbon monoxide (Thunberg tube); solid triangles, 6.8×10^{-4} M sodium cyanide (specimen tube); solid circles, 0.33 per cent oxygen (Thunberg tube).

time in specimen tubes or Thunberg tubes as appropriate. The results were expressed as the relative ATP content, *i.e.*, the inhibited level as a percentage of the control level (Table I). There was no correspondence between ATP content and rate of mitosis. However, it was noted that there is a very close concordance between the observed ATP content and the rate of respiration in the presence of these concentrations of inhibitor (values interpolated from Amoore (2, Fig. 5)). Thus it would be reasonable to conclude that the respiration rate determines the ATP level, but it is unjustifiable to suppose that the ATP level in turn controls the rate of mitosis, at least in pea root tips.

One of the results in Table I is particularly worth further investigation, and that is the finding that in 0.33 per cent oxygen, mitosis was able to continue at half the normal rate in air, while the ATP content had fallen to 3 per cent of the control level. In fact, the ATP level had fallen by 4 hours to only 1 per cent of the initial ATP content of freshly cut pea root tips. This raises once more in acute form the whole question of the origin of the energy required during the visible phase of mitosis under conditions of oxygen lack. One possibility is that a very small quantity of ATP might be able to serve, provided it was continuously

TABLE I

Effect of Inhibitors on Mitosis, ATP, and Respiration in Root Tips

(Values are percentages of values in air)

Inhibitor	Rate of mitosis	ATP content	Rate of respiration
0.33 per cent O_2	(50)	3	6
40 per cent CO	(50)	97	94
6.8×10^{-4} M HCN	(50)	37	18

regenerated by energy-yielding metabolic processes. That is, an unusually rapid turnover of ATP might perhaps compensate for its small quantity. However, direct experimental measurement of this turnover rate of ATP, when the absolute quantity is so small, would be difficult. An alternative approach is to consider the possible regeneration rate of ATP, by all the known metabolic pathways open under anaerobic conditions. The calculated regeneration rate of ATP can then be compared with the actual ATP content, to determine whether any compensatory acceleration of the turnover rate of ATP has occurred.

A step in this direction had been taken in an earlier paper (11) in which it was shown that under anaerobic conditions the evolution of carbon dioxide by root tips soon fell to only 8 per cent of the aerobic output. (It had been implicitly assumed that this anaerobic carbon dioxide arose from alcoholic fermentation of carbohydrate, which is the common anaerobic glycolytic pathway of plants.) It was pointed out

that such a small carbon dioxide evolution under anaerobic conditions would represent only about 1 per cent of the normal energy yield in air. However, other anaerobic metabolic pathways should perhaps be taken into consideration. For instance, there is the possible lactic fermentation of carbohydrate, familiar in animal metabolism, but rare in plants. Also, possibly a phosphagen breakdown could serve to regenerate ATP, a vital mechanism in animals, but not known to occur in plants.

The following experiment was designed to assess the contribution of all four pathways in pea root tips, *viz.*, aerobic respiration (by oxygen uptake), anaerobic alcoholic fermentation (by carbon dioxide evolution), lactic fermentation (by acid production), and phosphagen breakdown (by orthophosphate release). The results are shown in Table II, having been separately assessed for the first hour and for the subsequent 3 hours of the incubation, because it had been found previously (11) that marked changes occurred after 1 hour of anaerobiosis. The upper row of figures shows the actual exchange of metabolite, in units of mμmole/mg dry wt/hour. The oxygen consumption was large and steady. The anaerobic carbon dioxide evolution was weak, and decreased sharply after 1 hour. (After 3 hours the evolution gave way to a small gas uptake, whose cause is unknown.) The acid production was too small to measure, the figure shown being an upper limit. The small initial orthophosphate release increased substantially during the last 3 hours.

For purposes of comparison, the observed exchanges of metabolites were supposed to be coupled to the high energy esterification of phosphate (production of ~phosphate, or simply ~P). The coupling was assumed to have the full efficiency to be expected from biochemical considerations (23), *i.e.*, aerobically, 6 moles of ~P per mole of O_2; and anaerobically, 1 ~P per mole of alcoholic CO_2, 1.5 ~P per mole of lactic H^+, and 1 ~P per mole of PO_4^{3-}. These factors lead to the lower row of figures in Table II, showing the calculated esterification of ~phosphate which could be expected to accompany the observed metabolite exchanges in the upper row. The values show that during the first hour, the contributions by lactic fermentation and phosphagen breakdown were negligible, most of the energy probably being generated by alcoholic fermentation. However, after 1 hour the alcoholic fermentation also failed to produce more than a minute amount of energy.

The sum of the anaerobic sources of energy (foot of Table II) compares very unfavorably with the amount of energy normally available

TABLE II

Assessment of Major Energy-Yielding Metabolic Pathways in Excised Pea Root Tips

	Aerobic		Anaerobic					
	Oxygen uptake (*respiration*)		Carbon dioxide evolution (*alcoholic fermentation*)		Acid production (*lactic fermentation*)		Phosphate formation (*phosphagen breakdown*)	
	0–1 hour	1–4 hours	0–1 hour	1–3 hours	0–1 hour	1–4 hours	0–1 hour	1–4 hours
Actual metabolite exchange (mμmole/mg dry wt/hour)	−268	−304	+75	+13	<+2	<+2	+3	+12
Calculated ~phosphate esterification (mμmole/mg dry wt/hour)	1610	1820	75	13	<3	<3	3	12
					0–1 hour	1–4 hours		
					81	28		

− Represents consumption, and + represents production, of metabolite. At the foot of the table the total anaerobic ~phosphorylation has been summed for 0–1 hour and for 1–4 hours.

from aerobic respiration. During the first hour under anaerobic conditions, only about 5 per cent as much high energy phosphate generation would be expected to occur as under aerobic conditions, and subsequently the figure would fall to only 1.5 per cent (Table III). These values may be compared with the observed ATP contents in anaerobiosis, also expressed as percentages of the control values in air, and included in Table III (mean of 3 experiments). After 1 hour the ATP content was over three times as high as the calculated rate of ~P esterification. This excess is probably due to a short-lived episode of ATP production occurring about 1 hour after the root tips were placed in anaerobiosis (see Fig. 1). However, after 4 hours the observed ATP content agreed well with the calculated rate of ~P esterification. The actual ATP content is thus

TABLE III

Estimated ~P Esterification and Actual ATP Content in Root Tips

Time	~P esterification, anaerobic/aerobic	ATP content, anaerobic/aerobic
hrs.	%	%
0–1	5.0	17.8
1–4	1.5	1.4

closely proportional to its expected regeneration rate, which means that the turnover rate of the small remaining amount of ATP could not be much accelerated, if at all.

The conclusion from these experiments is that the ATP content of root tips agrees rather closely with its expected rate of regeneration via respiratory or glycolytic channels. However, the rate of mitosis bears no obligatory relationship to the ATP content. Furthermore, mitosis can continue slowly when the concurrent rate of energy production is only 1.5 per cent of normal.

Effect of Ferrous Coordination on Rate of Mitosis in Sea Urchin Eggs

This series of experiments deals with the application to sea urchin eggs of Amoore's (2) procedures for diagnosing the presence of the mitotic ferrous complex, to search for a possible dependence of mitosis upon the functioning of this complex, such as has been postulated to occur in pea root tips (2).

The diagnosis as applied to root tips consisted in showing that the rate of mitosis was not correlated with the rate of respiration, as influenced by oxygen lack, carbon monoxide, or hydrogen cyanide. On the contrary, the rate of mitosis was believed to depend upon the influence of these three coordinating agents on another ferrous complex, unconnected with respiration, but essential for mitosis. Now the manometric measurement of oxygen uptake in sea urchin eggs in the presence of carbon monoxide or hydrogen cyanide is rather uncertain, on account of CO oxidation (24) and HCN-stimulated respiration (25). Accordingly it would be preferable to make use of the ATP measurements, and to suppose that these closely reflect the true respiratory rate, as they do in root tips. (The ATP production of the sea urchin egg under anaerobic conditions is only about 5 per cent of that in the presence of oxygen, according to Cleland, quoted by Swann (26).)

The effects of various inhibitors of energy production on the rate of mitosis and the ATP level in sea urchin eggs have been tested in numerous experiments by Epel (1, and in preparation). In all instances, the progress of mitosis closely matched the ATP content, even when the fall in ATP content was brought about by agents such as 2,4-dinitrophenol, amytal, or antimycin A, which would not be expected to coordinate with the postulated mitotic ferrous complex. The progress of mitosis ceased completely, at any stage, when the ATP content had dropped to about 50 per cent of the normal level. Therefore mitosis in the sea urchin egg definitely requires a nearly normal level of ATP.

In view of this obligatory requirement for ATP, it became clear that any demonstration of the possible additional involvement of a mitotic ferrous complex would be much more difficult. Thus, considering the possible use of lowered oxygen tensions, it may be recalled that the respiration in pea roots was more dependent upon oxygen than was the mitosis (13). Hence in sea urchin eggs, a progressive reduction of oxygen tension would slow the respiration, lower the ATP level, and thereby bring about a stoppage of mitosis, long before any additional effect due to oxygen deprivation of the mitotic ferrous complex could be detected.

The same argument applies to the effects of hydrogen cyanide, because respiration in root tips was more sensitive to cyanide than was

mitosis (2). Nor does the light reversibility of the cyanide inhibition of mitosis help the diagnosis in sea urchin eggs, because cyanide inhibits the respiratory system just as effectively in the light as in the dark. Nevertheless, the effects of cyanide were tested by the present author on mitosis in sea urchin eggs, because the inhibitions through the ATP and ferrous systems might possibly have proved to be additive.

A range of concentrations of sodium cyanide (10^{-6} to 10^{-2} M) was tried, both in darkness and in light. The cyanide was added 102 minutes after fertilization, when most of the eggs were in late prometaphase and metaphase. The samples of eggs were fixed after a set time of exposure to cyanide, *viz.*, 60 minutes. The results are shown in Fig. 4. The rate of mitosis in sea urchin eggs was lowered to 50 per cent of the normal rate by 2.2×10^{-5} M hydrogen cyanide. This is only one-thirtieth of the cyanide concentration required to halve the rate of mitosis in pea root tips, *i.e.*, 6.8×10^{-4} M (2). On the other hand, it is close to the concentrations of cyanide required to halve the rate of respiration in *Arbacia* eggs, 7.4×10^{-6} M (25), or in pea root tips, 3.9×10^{-5} M (2). Furthermore, inhibition of mitosis by cyanide in the sea urchin eggs was the same, whether the inhibition was applied in darkness or in light.

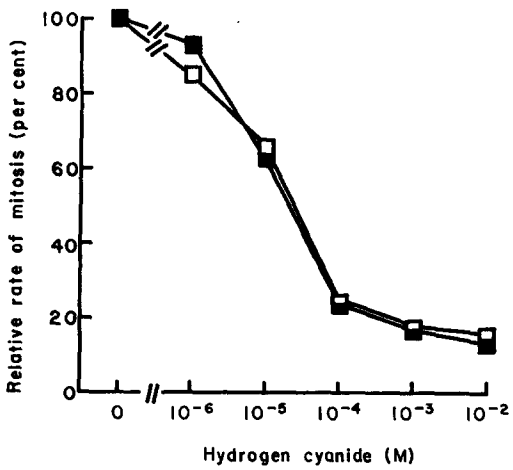

FIGURE 4 Effect of hydrogen cyanide on the relative rate of mitosis during the first division in sea urchin eggs. Solid squares, darkness; open squares, light. Inhibitor applied 102 minutes after fertilization. (In 10^{-3} or 10^{-2} M cyanide, the true rate of mitosis probably falls to zero; see text.)

This is in contrast with the situation in pea root tips, where light prevented the inhibition of mitosis by cyanide (2).

In the same experiment, 10^{-3} M cyanide was also applied to eggs 113 minutes after fertilization, when they were mainly in metaphase and anaphase, and also at 123 minutes, when they were in telophase and early interphase. The observed rates of mitosis were all between 16 and 20 per cent, in darkness or in light, agreeing closely with the rate observed when the same concentration of inhibitor was applied at 102 minutes (17 per cent, see Fig. 4). Therefore, all the phases of mitosis had an equal susceptibility to inhibition by cyanide.

It may be noted that even in the highest concentration of cyanide the rate of mitosis did not appear to fall below about 15 per cent (Fig. 4), when calculated over a 60-minute exposure. However, it has been shown by Epel (1), who calculated the rates at 3- to 5-minute intervals of exposure, that after the application of inhibitor, the cells are able to make about 5 or 10 minutes of progress at a reduced rate, before their development comes to a complete halt.

The experiment described above showed that the first division of the sea urchin egg was equally susceptible to 10^{-3} M hydrogen cyanide in darkness or in light. Now the first division is that of a very large and rather atypical cell, with astral spindles. Later divisions of the maturing embryo are of progressively smaller cells, whose spindles become anastral (27). The effect of 10^{-3} M cyanide was therefore also tested on 11-hour embryos, to ascertain whether they too exhibited the same susceptibility. Fig. 5 shows that the total number of mitotic figures visible in the embryos steadily increased between 11 and 15 hours, corresponding to the increasing number of cells in the embryo. However, when 10^{-3} M cyanide was present, no definite increase in the number of mitotic figures occurred either in darkness or in light. Therefore later divisions are just as susceptible as the first division to inhibition by cyanide. Furthermore, the lack of change in the number of mitoses shows that the mitoses were arrested for at least 4 hours.

If sea urchin eggs contained a mitotic ferrous complex, it should be most readily demonstrated by the application of carbon monoxide, because in pea root tips the mitotic ferrous complex was much more susceptible to carbon monoxide than

was the respiration (2). Thus, a low tension of carbon monoxide in darkness, or a higher tension in the presence of a weak light, could be expected to produce a slowing of mitosis, without affecting the respiration and the associated ATP generation. However, Epel's (1) paper shows no such selective effect of carbon monoxide in sea urchin eggs. In this and other experiments he has always found that any slowing of mitosis, even threshold slowing, was always accompanied by a drop in the ATP level.

The conclusion from these experiments and from Epel's data is that the progress of mitosis in

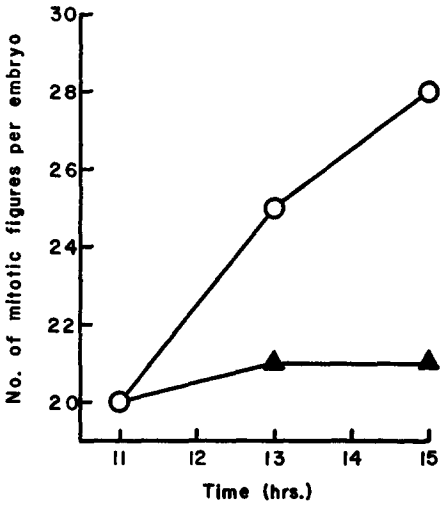

FIGURE 5 Effect of hydrogen cyanide on the number of mitotic figures in sea urchin embryos. Circles, control; triangles, 10^{-3} M cyanide (added to 11-hour embryos) in darkness or in light.

sea urchin eggs is obligatorily related to the ATP content, and stops completely when the ATP is reduced to about the 50 per cent level. No evidence could be found for the involvement of a non-respiratory ferrous complex during mitosis in sea urchin eggs.

DISCUSSION

The essential contrasts between the results obtained with pea root tips and with sea urchin eggs may be seen by referring to Table IV. There is fundamental agreement between Amoore and Epel that mitoses in both tissues can be arrested in progress by inhibitors of respiration (1, 10). This result is in contrast with the prevailing opinion expressed in reviews during the past decade, but was foreshadowed in some of the earlier literature. Epel (1) showed that the arrest of mitosis in sea urchin eggs is probably brought about by the stoppage of the mechanism for generating ATP, but the present paper shows that this explanation cannot apply to pea root tips. Conversely, whereas the arrest of mitosis in pea root tips was shown by Amoore (2) to be due to interference with a non-respiratory ferrous complex, no evidence could be found for the involvement of such a ferrous complex in sea urchin eggs (data of Epel (1), and present paper).

Hence, in these two tissues, two perfectly distinct biochemical mechanisms have been found to be responsible for the arrest of mitosis which results from the application of respiratory inhibitors. Now, older studies on the effects of oxygen lack on visible mitosis have been equivocal even regarding the factual question as to whether mitoses are arrested or not. As previously discussed (11), lack of knowledge about the degree of anaerobiosis achieved was probably responsible for the contradictory findings of different authors. In none of these investigations were the concurrent respiratory or ATP levels estimated, so that even when arrest was reported, it is not possible to tell which of the above biochemical mechanisms (if either) was responsible for this result. Therefore the whole matter of the comparative biochemistry of this phenomenon must continue to be regarded as an open question, until the biochemistry has been worked out in other tissues besides pea root tips and sea urchin eggs.

TABLE IV

Fundamental Contrasts between Roots and Eggs

	Pea root tips	Sea urchin eggs
Mitotic arrest by respiratory inhibitors?	Yes	Yes
This arrest due to stoppage of ATP generation?	No	Yes
This arrest due to interference with ferrous complex?	Yes	No

Bearing in mind the general biochemical tenet that energy transferred through the mediation of ATP is required for the vast majority of endergonic

cellular reactions and functions, it is most surprising to find that such a low ATP level (or ATP regeneration rate) as 1.5 per cent of normal is yet compatible with the continuation of the visible movements of mitosis in pea root tips (Table III). Although not formulated in terms of ATP, this problem had been foreseen by Bullough (3) and by Swann (6) as a consequence of their studies of the effects of inhibitors of energy metabolism upon the mitotic index. Bullough suggested that all the energy production necessary for mitosis was completed before prophase, and Swann elaborated this idea into the stimulating concept of a mitotic energy reservoir. The present author (11) felt that his own results with pea root tips supported the conclusions of Bullough and of Swann. However, with Epel's (1) proof that the energy reservoir hypothesis as applied to sea urchin eggs is very likely mistaken and superfluous, it seems timely to reexamine rather critically the belief that an energy reservoir may operate in pea root tips.

Numerous difficulties are encountered with the energy reservoir hypothesis as soon as one attempts to formulate the requirements that it must fulfill. First of all, there is no positive evidence as to its chemical identity. A suggestion of Swann (6) that high energy thiol-ester bonds are involved has apparently been abandoned. Next, its absolute concentration, if it were to be able to supply energy at near the normal rate for up to 4 hours, would be so high that it could hardly escape chemical detection. Thus the normal turnover rate of the ATP in root tips would have to be about once every 5 seconds, and the hypothetical energy-rich compound would have to have an initial concentration of the order of 1 molar to meet this requirement. Finally, in root tips, no chemical traces could be detected of the breakdown of any reservoir. It is difficult to imagine a major energy-yielding metabolic pathway or reaction which neither consumes oxygen, nor produces carbon dioxide, nor yields inorganic phosphate or indeed any acid product.

Thus both qualitative and quantitative considerations turn out to be rather unfavorable to the belief that a chemical energy reservoir may operate in root tips, to permit the continuance of mitotic movement after the stoppage of more than 98 per cent of the normal energy production on account of oxygen lack. Mazia (8), in discussing difficulties with the energy reservoir hypothesis, suggested that the reservoir may be more in the nature of a physical reservoir, built into the mitotic apparatus, which could supply the energy for anaphase movement at least. However, in both pea root tips (11) and sea urchin eggs (1) anaphase is no less susceptible to arrest by oxygen lack than are the other visible phases of mitosis. Hence the problem remains.

Nevertheless, Mazia (8) also put forward an alternative solution, to the effect that the energy requirements of "active mitosis" may in fact be so minute that the energy supply remaining even after the severest chemical inhibition may yet suffice to drive the chromosome movements. As Mazia points out, the dramatic movements of mitosis appear to our human way of thinking so obviously to require work that the concept is not easily credible that these movements actually consume very little energy. Nevertheless, the energy required to move the chromosomes from the equator to the poles during anaphase in pea root tip cells may be approximately estimated by applying Perrin's extension of Stokes' law (28) to the chromosomes. The data required are the number and size of the chromosomes ($4n = 28$, about 0.4 μ in diameter and 4 μ long in pea root tips (29)); the rate of movement of the chromosomes (1.8 μ/minute in pea endosperm (30)): and the cytoplasmic viscosity (0.25 poise in bean shoot endodermal cells (31)). The result of the estimation is that the expenditure of only about 0.8×10^{-13} erg/second/cell would suffice to power the anaphase movement of chromosomes.

To place this minute quantity of energy in perspective it is necessary to calculate how much energy could be available to the cell under aerobic or anaerobic conditions. The data required are the expected rate of esterification of high energy phosphate in the pea root tips (1750 mμmole $\sim$P/mg dry wt/hour in air, and 28 mμmole $\sim$P/mg dry wt/hour in nitrogen; data from Table II above); the free energy of ATP hydrolysis in living cells (-11.5 kcal/mole $\sim$P (32)); the number of cells per 2.0 mm pea root tip (152,000 (33)); and the dry weight of 1 root tip (average 0.18 mg). The result of the calculation is that the energy available from respiration in air would be 2.7×10^{-4} erg/second/cell. This is over 10^9 times as much energy as would theoretically be required to power anaphase movements. Even under nitrogen the energy available to the root tips from anaerobic metabolism would be $4.4 \times$

10^{-6} erg/second/cell. This is still over 10^7 times as much energy as the chromosome movements during anaphase were calculated to require.

For comparison, the amount of energy required for the synthesis of several polymeric cellular constituents may be calculated. The pea root tip (1.6 mm) is composed almost entirely of meristematic cells (34), which have an average mitotic cycle of 16 hours at 25°C (35). Hence the entire substance of the root tip may be considered to be reduplicated every 16 hours. Heyes (33) found that the 2.0-mm pea root tip contains 7.5 $\times$ 10^{-5} gm protein, 10.4 $\times$ 10^{-6} gm ribonucleic acid (RNA), and 4.2 $\times$ 10^{-6} gm deoxyribonucleic acid (DNA). The monomer residue molecular weights are taken to be 120, 320, and 310 for amino acid, ribonucleotide, and deoxyribonucleotide respectively. The activation of each residue requires the consumption of 2 high energy phosphate bonds (36–38). From these data it may be calculated that the synthesis (polymerization) of protein alone must consume at least 25 per cent of the total aerobic energy yield of the root tip. The synthesis of RNA would need about 1 per cent and of DNA about ½ per cent. (These are minimal values, and do not include the energy needed for synthesizing the monomers.)

Now the synthetic activities of dividing cells are probably maximal during interphase and fall to a minimum during mitosis (8). This is certainly true of DNA synthesis (39), and probably also true of RNA synthesis (40) and protein synthesis (41). Hence the demand for energy will presumably be greatest during interphase and smallest during mitosis. Therefore the common observation that interference with the respiratory generation of energy in dividing cells inhibits interphase much more drastically than mitosis (2, 3, 6, 7, 13) receives a simple explanation, without resort to the energy reservoir hypothesis.

These calculations support the alternative hypothesis that the energy required for the visible movements of active mitosis may be so minute that the energy supply is never likely to be limiting, even in severely inhibited cells. Hence it seems to be quite unnecessary to invoke the energy reservoir hypothesis to explain the experimental observations on mitosis in pea root cells. Considering that the hypothesis is probably inapplicable even to the very material, sea urchin eggs, for which it was originally formulated by Swann (4) (see Epel (1)), it may be wondered whether the concept of the energy reservoir retains any further significance at the present time. As a working hypothesis in ordering the often confusing data of this difficult topic, the concept has been most valuable and stimulating; however, until positive evidence of its physicochemical identity is forthcoming, the concept of a specific energy reservoir for mitosis should perhaps be held in abeyance.

The present work and that of Epel (1) leave two important problems unanswered. Why should so high an ATP level as 50 per cent of normal be necessary for the continuance of mitosis in sea urchin eggs, whereas only 1.5 per cent suffices in pea root tips? Also why is the unidentified ferrous complex required during mitosis in pea roots, but seemingly unnecessary in sea urchin eggs?

Regarding the former question, there are several reasons for considering that the movements of chromosomes during mitosis may be quite analogous to the movements of muscular contraction (42, 43). The mitotic movements in the sea urchin egg cease when the ATP level is lowered to about 50 per cent of normal by respiratory inhibition (1). It may be recalled that an analogous phenomenon occurs in rabbit muscle, when the postmortem decrease in ATP level is accompanied by a parallel loss in contractility, until the muscle becomes completely immobile and inelastic, while still containing about 30 per cent of the normal ATP (44). It is interesting to note that the absolute ATP concentrations present in muscle and in eggs, when contraction and mitosis cease, are nearly the same, *i.e.*, 1.6 and 1.3 $\times$ 10^{-3} mole ATP per kg fresh weight respectively (assuming the diameter of the sea urchin egg to be 80 μ).

Hoffmann-Berling (45) showed that a much lower concentration of ATP (5 $\times$ 10^{-6} M) would act specifically to set glycerol-extracted models of *Tachycines* sperm flagella in motion, provided the models were maintained in a "plastic" condition. This could be achieved by various non-specific agents such as pyrophosphate, Germanin, or simply a raised ionic strength. Indeed, excess of ATP itself acted as a softening agent. Hence the finding of a high threshold concentration of ATP (10^{-3} M) to permit contraction in muscles or mitosis in eggs may not represent an energetic limitation so much as a mechanical one.

In root tips under 0.33 per cent oxygen the absolute level of ATP fell to only 3 $\times$ 10^{-6} M, yet mitotic movements continued. This is about

the same concentration of ATP as sufficed to cause flagellar movement. However, total removal of oxygen does arrest mitosis in roots. Therefore it may be wondered whether oxygen may be acting in the role of a "plasticizer" in root tip mitoses, whereas excess ATP itself may fill this role in muscles and sea urchin eggs.

Regarding the question of the failure to demonstrate the involvement of the unidentified ferrous complex in sea urchin eggs, whereas it plays some essential part in conjunction with oxygen in root tip mitoses, there is little to add. The diagnostic method depended on the light-reversible inhibition of mitosis by cyanide, which gave a positive result in pea root tips, but not in sea urchin eggs. Recently Hirokawa (46) found that a catalase-rich fraction from *Chlorella* cells showed a light-reversible inhibition of hydrogen peroxide decomposition in the presence of 10^{-5} M sodium azide. However, the inhibition of beef liver catalase by azide was quite unaffected by light. This phenomenon shows several parallels with the problem in hand.

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REFERENCES

1. EPEL, D., The effects of carbon monoxide inhibition on ATP level and the rate of mitosis in the sea urchin egg, *J. Cell Biol.*, 1963, **17**, 315.
2. AMOORE, J. E., Participation of a non-respiratory ferrous complex during mitosis in roots, *J. Cell Biol.*, 1962, **13**, 373.
3. BULLOUGH, W. S., The energy relations of mitotic activity, *Biol. Rev.*, 1952, **27**, 133.
4. SWANN, M. M., The mechanism of cell-division. A study with carbon monoxide on the sea-urchin egg, *Quart. J. Micr. Sc.*, 1953, **94**, 369.
5. BULLOUGH, W. S., and JOHNSON, M., The energy relations of mitotic activity in adult mouse epidermis, *Proc. Roy. Soc. London, Series B*, 1951, **138**, 562.
6. SWANN, M. M., The control of division: a review. I. General mechanisms, *Cancer Research*, 1957, **17**, 727.
7. STERN, H., Mitosis and metabolic organization, *Bot. Rev.*, 1959, **25**, 351.
8. MAZIA, D., Mitosis and the physiology of cell division, *in* The Cell, (J. Brachet and A. E. Mirsky, editors), New York, Academic Press, Inc., 1961, **3**, 77.
9. BULLOUGH, W. S., The control of mitotic activity in adult mammalian tissues, *Biol. Rev.*, 1962, **37**, 307.
10. AMOORE, J. E., Arrest of mitosis in roots by oxygen-lack or cyanide, *Proc. Roy. Soc. London, Series B*, 1961, **154**, 95.
11. AMOORE, J. E., Dependence of mitosis and respiration in roots upon oxygen tension, *Proc. Roy. Soc. London, Series B*, 1961, **154**, 109.
12. UTKIN, I. A., Experimental study of mitoses in the corneal epithelium of eyes incubated "in vitro," *Path. et biol., Semaine hôp.*, 1961, **9**, 519.
13. AMOORE, J. E., Oxygen tension and the rates of mitosis and interphase in roots, *J. Cell Biol.*, 1962, **13**, 365.
14. STREHLER, B. L., and TOTTER, J. R., Firefly luminescence in the study of energy transfer mechanisms, *Arch. Biochem. and Biophysics*, 1952, **40**, 28.
15. WAHL, R., and KOZLOFF, L. M., The nucleoside triphosphate content of various bacteriophages, *J. Biol. Chem.*, 1962, **237**, 1953.
16. UMBREIT, W. W., BURRIS, R. H., and STAUFFER, J. F., Manometric Techniques, revised edition, Minneapolis, Burgess Publishing Co., 1957.
17. ALLEN, R. J. L., The estimation of phosphorus, *Biochem. J.*, 1940, **34**, 858.
18. MAZIA, D., and ZIMMERMAN, A. M., SH compounds in mitosis. II. The effect of mercaptoethanol on the structure of the mitotic apparatus in sea urchin eggs, *Exp. Cell Research*, 1958, **15**, 138.
19. ALBAUM, H. G., The role of phosphorus in the metabolism of plants, *in* The Biology of Phosphorus, (L. F. Wolterink, editor), Michigan State College Press, 1952, 37.
20. ŠEBESTA, K., and ŠORM, F., Veränderungen des Gehaltes an Adenosintriphosphosäure im Verlaufe der Keimung der Gartenbohne, *Collection trav. chim. tchécoslov.*, 1956, **21**, 1047.
21. CHERRY, J. H., and HAGEMAN, R. H., Separation and identification of soluble nucleotides from etiolated corn seedlings as a function of growth, *Plant Physiol.*, 1960, **35**, 343.
22. BULLOUGH, W. S., and LAURENCE, E. B., The study of mammalian epidermal mitosis *in vitro*. A critical analysis of technique, *Exp. Cell Research*, 1961, **24**, 289.
23. BALDWIN, E., Dynamic Aspects of Biochemistry,

3rd edition, London, Cambridge University Press, 1957.

24. BLACK, R. E., and TYLER, A., Effects of fertilization and development on the oxidation of carbon monoxide by eggs of *Strongylocentrotus* and *Urechis* as determined by use of C^{13}, *Biol. Bull.*, 1959, **117**, 443.
25. ROBBIE, W. A., The effect of cyanide on the oxygen consumption and cleavage of the sea urchin egg, *J. Cell. and Comp. Physiol.*, 1946, **28**, 305.
26. SWANN, M. M., The mechanism of cell-division: The action of 2,4 dinitrophenol and certain glycolytic inhibitors on the sea urchin egg, *Proc. Roy. Phys. Soc. Edinburgh*, 1955, **24**, 5.
27. HARRIS, P., Some structural and functional aspects of the mitotic apparatus in sea urchin embryos, *J. Cell Biol.*, 1962, **14**, 475.
28. TANFORD, C., Physical Chemistry of Macromolecules, New York, John Wiley & Sons, Inc., 1961.
29. SANSOME, E. R., Segmental interchange in *Pisum* II, *Cytologia*, 1933, **5**, 15.
30. BAJER, A., and MOLÈ-BAJER, J., Endosperm, material for study on the physiology of cell division, *Acta Soc. Bot. Polon.*, 1954, **23**, 69.
31. HEILBRONN, A., Zustand des Plasmas und Reizbarkeit. Ein Beitrag zur Physiologie der lebenden Substanz, *Jahrb. wissensch. Bot.*, 1914, **54**, 357.
32. BURTON, K., Energy of adenosine triphosphate, *Nature*, 1958, **181**, 1594.
33. HEYES, J. K., Nucleic acid changes during cell expansion in the root, *Proc. Roy. Soc. London, Series B*, 1960, **152**, 218.
34. BROWN, R., and BROADBENT, D., The development of cells in the growing zones of the root, *J. Exp. Bot.*, 1950, **1**, 249.
35. BROWN, R., The effect of temperature on the durations of the different stages of cell division in the root tip, *J. Exp. Bot.*, 1951, **2**, 96.
36. BERG, P., Specificity in protein synthesis, *Ann. Rev. Biochem.*, 1961, **30**, 293.
37. ABRAMS, R., Nucleic acid metabolism and biosynthesis, *Ann. Rev. Biochem.*, 1961, **30**, 165.
38. KORNBERG, A., Biologic synthesis of deoxyribonucleic acid, *Science*, 1960, **131**, 1503.
39. WIMBER, D. E., Duration of the nuclear cycle in *Tradescantia paludosa* root tips as measured with H^3-thymidine, *Am. J. Bot.*, 1960, **47**, 828.
40. TAYLOR, J. H., Nucleic acid synthesis in relation to the cell division cycle, *Ann. New York Acad. Sc.*, 1960, **90**, 409.
41. PRESCOTT, D. M., and BENDER, M. A., Synthesis of RNA and protein during mitosis in mammalian tissue culture cells, *Exp. Cell Research*, 1962, **26**, 260.
42. HOFFMANN-BERLING, H., Die Bedeutung des Adenosintriphosphat für die Zell- und Kernteilungsbewegungen in der Anaphase, *Biochim. et Biophysica Acta*, 1954, **15**, 226.
43. MAZIA, D., CHAFFEE, R. R., and IVERSON, R. M., Adenosine triphosphatase in the mitotic apparatus, *Proc. Nat. Acad. Sc.*, 1961, **47**, 788.
44. BORBIRO, M., and SZENT-GYÖRGYI, A., On the relation between tension and ATP in cross-striated muscle, *Biol. Bull.*, 1949, **96**, 162.
45. HOFFMANN-BERLING, H., Geisselmodelle und Adenosintriphosphat (ATP), *Biochim. et Biophysica Acta*, 1955, **16**, 146.
46. HIROKAWA, T., Light-induced oxygen evolution caused by hydrogen peroxide in catalase-inhibited *Chlorella* cells, *Plant and Cell Physiol.*, 1962, **3**, 197.