THE EFFECT OF CHANGES IN THE RESPIRATORY METABOLISM UPON GENOME ACTIVITY

A Correlation between Induced Gene Activity and an Increase in Activity of a Respiratory Enzyme

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ABSTRACT

The in vitro regression of experimentally induced chromosome puffs was investigated in explanted salivary gland chromosomes of *Drosophila hydei*. It was observed that the regression of the puffs 2-32A, 2-36A, 2-48C, and 4-81B is accelerated if substrates for the respiratory metabolism are supplied to the cells. A similar effect can be produced by addition of KCN or oligomycin to medium in which intact salivary glands are incubated. The acceleration of puff regression by these substances occurs not only if the puff-inducing stimulus is removed but as well under conditions in which the stimulus is maintained. Regression of the puffs 2-32A, 2-36A, and 4-81B is inhibited if cycloheximide is present in the incubation medium. Chloramphenicol has no effect on puff regression. Measurements on nicotinamide adenine dinucleotide-dehydrogenase activity in homogenates of salivary glands revealed an increase in enzyme activity of 41%. Maximum increase is attained at 30 min after the induced puffs have reached their maximum size. The increase in enzyme activity does not occur if the glands are kept in a medium containing either actinomycin D or cycloheximide. Chloramphenicol does not inhibit the increase in enzyme activity. The possible relationship between puff activity and its control as a result of changes in the respiratory metabolism is discussed.

INTRODUCTION

Puffs in the polytene chromosomes of Diptera are considered to represent morphological manifestations of gene activity (Beermann, 1967). Over the years a variety of agents has been reported to influence the pattern of puffs in the polytene chromosomes in several ways by inducing the appearance of new puffs and/or the disappearance of existing ones (Ritossa, 1963, 1964; Clever, 1964; Kroeger, 1963; Berendes, 1967; Ashburner, 1970).

Recently, it was demonstrated that substances which interfere in a specific way with steps in the cellular respiration chain have an effect on the activity of a particular group of chromosome loci (Leenders and Berendes, 1972). It was indicated that these loci became active in instances in which the respiratory metabolism could not meet the demands of the cell. The observations suggested that the control of activity of the chromosome loci finds its origin in, or is at least related with, a mechanism which controls the respiratory metabolism. If this interpretation were correct, it could be assumed that the particular group of puffs synthesizes products which have a definite message for the respiratory metabolism. It could further be

suggested that once these products become available to the cell, the respiratory metabolism would be stimulated to meet the demands of the cell. If this situation would be established, further activity of the chromosome loci would not be required anymore, and as a consequence the information-producing loci should become inactive again.

So far, little attention has been paid to the phenomenon of regression and the possible mechanisms involved in its control. It is known that the puffs which appear upon a sudden increase in the demands on the respiratory metabolism, e.g. as a consequence of a rise in environmental temperature from 25° to 35°C, disappear after a certain period of activity if the animals are maintained at the higher temperature (Berendes and Holt, 1964; van Breugel, 1966). The same effect, puff regression, is observed if the larvae are returned to the original temperature (Berendes, 1969).

This report presents the results of experiments designed to test the assumption whether or not regression of these puffs can occur as a consequence of the reestablishment of the balance between the cellular demand and the output of the respiratory metabolism. Puff regression was either accelerated, inhibited, or delayed by providing the cells, after induction of the puff, with substances supporting or inhibiting the respiratory metabolism. In addition to these experiments, quantitative determinations of one of the respiratory enzymes, the nicotinamide adenine dinucleotide (NADH)-dehydrogenase, were performed on homogenates of cells which had been submitted to some of the experimental conditions.

MATERIALS AND METHODS

All experiments were performed with larval salivary glands of *Drosophila hydei*. The isolation of the glands, the conditions for short-term incubation, the preparation of squashes for analysis of the chromosomal puffing pattern, and the measurements on puff size were carried out as described previously (Leenders and Berendes, 1972).

Puff activity was induced at the loci 2-32A, 2-36A, 2-48C, and 4-81B by a temperature treatment involving a transfer of intact larvae from 23(±1)°C (room temperature) to 37(±1)°C. After 30 min at 37°C, the salivary glands were isolated and incubated at room temperature. Puff size was measured at 5, 10, 20, 25, 30, and 35 min after the onset of incubation at 23°C.

Similar series of experiments were performed in incubation media containing either oligomycin (saturated solution) (Sigma Chemical Co., St. Louis, Mo.), 10^{-2} M KCN (E. Merck, A. G., Darmstadt, West Germany), or 10^{-2} M sodium malate (Sigma Chemical Co.) + 10^{-2} M sodium succinate (Fluka AG, Basel, Switzerland). All incubations were performed at 23°(±1)°C.

Puff regression was also investigated after in vitro induction of puff activity accomplished by placing salivary glands dissected from animals kept at 23°C in a medium at 35°C. After 30 min of incubation at the higher temperature, the medium containing one gland of a larva was supplied with either oligomycin, KCN, or malate plus succinate (concentrations as in the experiments described above), whereas the contralateral gland received the same volume of standard medium without additives. The sizes of two of the aforementioned puffs, 2-48C and 4-81B, were measured at 50 min after addition of the substances and compared with those of the puffs in the sister gland after the same period of incubation in standard medium. During the incubation the temperature was kept at 35(±1)°C.

Puff regression was also investigated in a series of experiments in which the puffs were induced in vitro by the presence of 10^{-3} M 2,4-dinitrophenol (DNP) in the incubation medium. The incubation was carried out at 23°C and puff regression was determined in the presence of DNP at 50 min after addition of the substances mentioned before.

In order to investigate the effects of inhibitors on protein synthesis, cycloheximide, and chloramphenicol, long-term incubations were performed in a medium described by Shields and Sang (1970) and modified according to Poels (1972).

In this medium, the salivary glands remain unaltered with respect to their physiological response over a period of at least 12 hr (Poels, 1972). Salivary glands were incubated in this medium at 35(±1)°C and the appearance and regression of the four puffs were followed at successive time intervals (up to 4 hr) after the onset of incubation. The normal appearance and regression of the puffs was compared with that in the same medium after addition of 4 μg/ml cycloheximide or 100 μg/ml chloramphenicol. In these experiments the temperature was kept continuously at 35°C.

Preparation and Assay of NADH-Dehydrogenase

NADH-dehydrogenase activity was assayed in homogenates of salivary glands displaying experimentally induced puffs. The activity in these homogenates was compared with that in homogenates of glands in which the puffs had not been activated.

For determination of NADH-dehydrogenase activity, 40 g of larvae were divided into two batches of 20 g each (each batch consisted of approximately 4000 larvae). One of the batches was submitted to a 2 hr CO_2 anaerobiosis treatment (see Leenders and

Berendes, 1972) which was followed by recovery of the animals in air. This treatment results in the induction of the specific puffs within 30 min after the onset of the recovery period. The other batch of larvae was used as control. The salivary glands of both batches of larvae were isolated according to the method described by Boyd et al. (1968). Salivary glands from CO_2-treated animals were isolated at 30 min, 60 min, 90 min, or 120 min after the onset of the recovery period and before the recovery period was started. From the isolated salivary glands, 50 mg were homogenized in 5 ml 0.1 M Tris-HCl containing 0.003 M deoxycholate (pH 8.0) by sonication with a Branson sonifier (10 sec) according to Hatefi et al. (1961). After sonication, KCl was added to a final concentration of 1 M.

The assay of NADH-dehydrogenase was performed by following the rate of NADH oxidation spectrophotometrically at 366 mμ. The assay system was composed of 1.65 ml 0.1 M Tris-HCl (pH 8.0 at 25°C), 0.2 ml homogenate, and 10–40 μl of 0.01 M NADH. The reaction was started by addition of 0.25 ml 0.01 M K_3 ($Fe[CN]_6$). V_{max} of the NADH-dehydrogenase was calculated on the basis of a Lineweaver-Burk plot (Dixon and Webb, 1964). Protein quantity was determined according to Lowry et al. (1951).

For the study of the effect of actinomycin D (4 μg/ml), cycloheximide (4 μg/ml), and chloramphenicol (100 μg/ml) on NADH-dehydrogenase activity after a period of 85 min recovery from a CO_2 treatment, the salivary glands were isolated at 23°C (room temperature) in a medium containing these antibiotics. If *de novo* synthesis of NADH-dehydrogenase should result from the CO_2 treatment, this synthesis probably would be interrupted or reduced by isolation of the glands at the usual temperature of 4°C (see Boyd et al., 1968). Moreover, the inhibitors of protein synthesis should be active in this procedure from the moment at which the glands are released from the animals.

RESULTS

If salivary glands displaying puff activity at the loci 2-32A, 2-36A, 2-48C, and 4-81B resulting from an in vivo temperature treatment of 30 min are isolated and maintained in an incubation medium at 23°C, regression of the puffs is completed after 30–35 min. Regression is accelerated when malate (10^{-2} M) plus succinate (10^{-2} M) are supplied to the incubation medium. Under this condition, puff 4-81B has completely regressed after 20 min and puff 2-48C after 30 min (see Fig. 1). This result indicated that a support of the respiratory metabolism with substrates accelerates puff regression, which is already occurring as a consequence of the withdrawal of the stimulus (a temperature treatment). In order to test whether this effect results from a stimulation of the energy (adenosine triphosphate [ATP])-providing system in a more general sense, regression was studied under conditions in which the ATP production was inhibited by oligomycin. Oligomycin (saturated solution) in the incubation medium did affect the rate of puff regression in the same manner as was observed in the presence of substrates (Fig. 1). Puff regression was accelerated. This indicated that the production of ATP by the terminal respiration as such has no obvious relationship to the mechanism controlling the activity of the specific puffs. This indication was further supported by the effect of addition of KCN (10^{-2} M) to the incubation medium, which accelerated

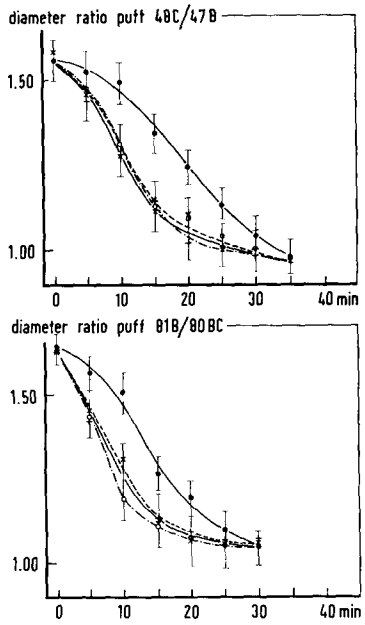

FIGURE 1 The effect of the presence of malate plus succinate, oligomycin, and KCN in the incubation medium upon the regression of two puffs, 2-48C and 4-81B, after a temperature treatment. ●——●, regression without any additive; ○—•—○, regression in the presence of 10^{-2} M malate plus 10^{-2} M succinate; +---+, regression in the presence of oligomycin (saturated); ×——×, regression in the presence of 10^{-2} M KCN. Ratio 2-48C/47B is obtained by measurements on the diameter of region 2-48C (puff locus) and that of 47B (nonpuffed band). The same applies for the ratio 4-81B/80BC. Each point represents an average of at least 10 measurements. Standard errors are presented for each average of the control and for the highest and lowest averages in the presence of additives.

puff regression too. It should be pointed out that KCN and the substrates malate and succinate may have a common effect on the respiratory metabolism, inasmuch as the respiratory chain should be shifted to a more reduced state by both additions to the medium. In addition to an increase in reduced intermediates of the chain, substrate requirement should be reduced under these conditions.

The results so far presented find support from a series of experiments in which the puffs were induced in vitro (temperature treatment at 35°C or by addition of 10^{-3} M DNP) and the medium was supplied with either the substrates malate and succinate, KCN, or oligomycin at 30 min after onset of the inductive treatment. At 20 min after the administration of these compounds to the medium, the diameters of the puffs were measured and compared with those in control (sister-) glands which had been kept over 50 min in the standard medium. In both instances the temperature of the media was continuously kept at 35°C, or DNP remained present during the whole incubation period. Table I reveals that the addition of substrates, oligomycin, or KCN results in a significant reduction of the puff diameters as compared with the controls.

In order to establish whether or not the regression of the experimentally induced puffs is dependent on the capacity of the cells for RNA and protein synthesis, regression was studied in the presence of inhibitors. It has already been described that actinomycin D (20 μg/ml) injected into larvae at 10 min after onset of a temperature treatment results in only a partial regression of the puffs

TABLE I

Effects of Substrates, KCN, and Oligomycin upon Regression of the Puffs 2-48C and 4-81B under Conditions of Continued Temperature or DNP Treatment (Incubation of Single Glands in 18 μl Medium)

Stimulus during 50 min	Injection into the medium after 30 min	Diameter ratio 48C/47B	*P* value*	Diameter ratio 81B/80BC	*P* value*	Number of experiments	Number of nuclei
T shock 25°–35°C	2 μl medium	1.52		1.50		3	15
			≤0.001		≤0.001		
T shock 25°–35°C	2 μl medium + 10^{-1} M malate + 10^{-1} M succinate	1.30		1.17		3	15
T shock 25°–35°C	2 μl medium	1.50		1.51		3	15
			≤0.001		≤0.001		
T shock 25°–35°C	2 μl medium + 10^{-1} M KCN	1.22		1.20		3	15
T shock 25°–35°C	2 μl medium	1.49		1.50		3	10
			≤0.001		≤0.001		
T shock 25°–35°C	2 μl medium + oligomycin (saturated solution)	1.24		1.25		3	10
10^{-3} M DNP	2 μl medium	1.51		1.50		3	15
			≤0.001		≤0.001		
10^{-3} M DNP	2 μl medium + 10^{-1} M malate + 10^{-1} M succinate	1.24		1.25		3	15
10^{-3} M DNP	2 μl medium	1.44		1.42		3	10
			≤0.005		≤0.009		
10^{-3} M DNP	2 μl medium + 10^{-1} M KCN	1.19		1.16		3	10

* Analysis of variance showed the data to be homogeneous within and between glands of a given experimental set. *P* values refer to comparisons of controls with experimental sets by a *t* test.

(Berendes, 1968). The experiments to be described have been performed on glands maintained in a culture medium composed of substrates for the Krebs' cycle, amino acids, and bovine serum (Poels, 1972). Salivary glands kept for 12 hr in this medium display a characteristic response to a temperature treatment in showing the development of the specific puffs. It appeared that upon incubation of the glands in excess of medium, the maximum size of the puffs was attained after 80 min of temperature treatment, whereas in a simple Ringer's solution this size is reached within 45 min.

If glands dissected from larvae reared at 23°C are transferred to culture medium which contains cycloheximide (4 μg/ml) or chloramphenicol (100 μg/ml) and has a temperature of 35°C, the specific puffs develop. The puffs 2-32A, 2-36A, and 4-81B attain a size which is identical to that in control (contralateral) glands incubated in culture medium without antibiotics for the same period of time. The same applies for puff 2-48C, if its formation is followed in the presence of chloramphenicol. However, this puff displays a behavior different from that of the other puffs if its formation is studied in a medium supplied with cycloheximide: under this condition, puff 2-48C never reaches a size equivalent to that in control (contralateral) glands which are incubated without the antibiotic.

At 35°C, the onset of puff regression can be observed after 2 hr of incubation without antibiotics (which is approximately 40 min after the puffs have attained their maximum size). The puffs have almost completely disappeared between 2 and 3 hr after regression began. A similar behavior was observed in glands incubated with chloramphenicol. However, a different behavior of three of the puffs is observed if cycloheximide is present in the medium. In this case the puffs 2-32A, 2-36A, and 4-81B do not start regression at the same time as in the controls, but puff size remains unchanged, or is slightly reduced after 3 hr or longer incubation (Fig. 2). Puff 2-48C did not reveal a similar behavior. This puff regressed in spite of the presence of cycloheximide in the medium, at a rate almost identical to that of the controls.

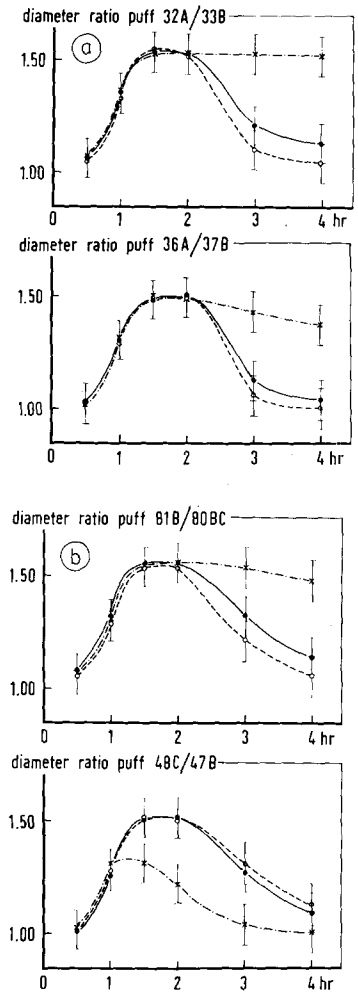

FIGURE 2 The effect of the presence of cycloheximide and chloramphenicol in the long-term incubation medium upon development and regression of the puffs 2-32A, 2-36A, 2-48C, and 4-81B. •——•, development and regression of the puffs without any additive; ×—•—×, in the presence of cycloheximide; ○---○, in the presence of chloramphenicol. Puff ratios and standard errors as in Fig. 1.

Measurements on NADH-Dehydrogenase Activity

The high temperature which stimulates puffing can be expected to increase demands on the respiratory metabolism, and the question arises whether or not the puff response is relevant for the adaptation of the respiratory mechanism to this change. That question was approached by measuring the activity of NADH-dehydrogenase which is one of the enzymes that could have a rate-limiting position with regard to substrate utilization by the mitochondrial respiratory chain.

In all experiments in which NADH-dehydrogenase activity was assayed, the size of one of the

specific puffs, 4-81B, was measured in parallel experiments with intact salivary glands. It should be emphasized that this particular puff was chosen as representative of the entire group of puffs rather than on the basis of an expected specific relationship between this puff and enzyme activity. The results of the average of three series of measurements of puff size and NADH-activity are recorded in Fig. 3. The values recorded for NADH-dehydrogenase-activity represent V_{max}. These values were obtained by calculation of the regression line on the basis of the specific activity at five different NADH concentrations according to a Lineweaver-Burk plot. From Fig. 3 it can be concluded that the V_{max} of the NADH-dehydrogenase begins to increase 20 min after the onset of puff formation and attains a maximum at approximately 30 min after the puff size has reached its maximum value. Under the conditions applied, V_{max} reaches a value of 140% as compared with the controls. The enzyme activity remains at approximately that level for 30 min and then decreases. Because the enzyme activity was measured as V_{max}, the increase in activity values indicates a net increase in enzyme quantity rather than activation of already present inactive enzyme molecules. This indication was further tested by performing measurements on NADH-dehydrogenase activity in homogenates of glands which were, after an anaerobiosis treatment, incubated in vitro with actinomycin D, cycloheximide, or chloramphenicol. The results of these measurements are presented in Table II. It appears that cycloheximide and actinomycin D inhibit the increase in V_{max} after the anaerobiosis treatment, whereas chloramphenicol fails to do so. These data, therefore, lend support to the idea that the

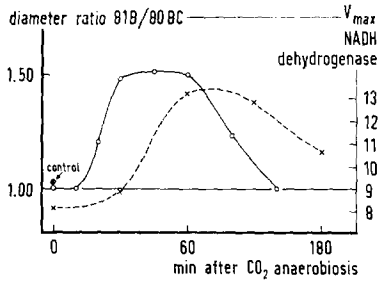

FIGURE 3 Development and regression of puff 4-81B (representative of the whole group of puff loci) and the course of the activity (V_{max} of the NADH-dehydrogenase) during 180 min after CO_2 anaerobiosis. ○——○, development and regression of puff 4-81B; ×---×, V_{max} NADH-dehydrogenase. Each V_{max} point represents an average of 3 V_{max} values obtained from Lineweaver-Burk plots. The V_{max} averages after 60 and 90 min are significantly different from the control value as in the figure indicated (t test).

TABLE II

V_{max} Values for NADH-Dehydrogenase Attained under In Vitro Conditions during Recovery from Anaerobiosis in the Presence of Actinomycin D, Cycloheximide, and Chloramphenicol

Source of homogenate	V_{max} in μmole/min/mg protein	% of increase in V_{max} over controls	Puffing pattern
(*a*) Control glands not submitted to anaerobiosis	9.14	—	No specific puffs present
(*b*) Glands at 85 min after the onset of recovery from anaerobiosis	11.70	28 ($P \leq 0.05$)	Small specific puffs present
(*c*) Same as *b* in the presence of actinomycin D (4 μg/ml)	9.23	1	Very small specific puffs present
(*d*) Same as under *b* in the presence of chloramphenicol (100 μg/ml)	11.79	29 ($P \leq 0.05$)	Small specific puffs present
(*e*) Same as under *b* in the presence of cycloheximide (4 μg/ml)	9.41	3	Large specific puffs present, puff 2-48C regresses

Measurements were performed on homogenates of whole salivary glands.
Each value represented is an average of three experiments. The specific puffs are located at 2-32A, 2-36A, 2-48C, and 4-81B. P values refer to comparisons of controls with experimental sets by a t test.

increase in NADH-dehydrogenase activity is a consequence of a *de novo* synthesis of this enzyme or part of it.

DISCUSSION

From the data presented it is evident that the regression of puffs, which seem to become active as a consequence of a temporary incompetence of the intracellular respiratory metabolism to meet the demands of the cell, is accelerated in all instances in which the respiratory metabolism is supported by exogenous substrates. This result as well as the results presented in a previous paper (Leenders and Berendes, 1972) suggests that in a situation in which the respiratory chain shifts to a more oxidized state, a factor (or factors) is released which is capable of initiating the activity of specific genome loci, e.g., 2-32A, 2-36A, 2-48C, and 4-81B. The observed effects of KCN and oligomycin are compatible with this idea because these substances inhibit the respiratory metabolism with the result that intermediates of the chain become more reduced. It is by no means excluded that this shift of the respiratory chain to a more reduced state has consequences for the utilization of substrates and eventually for the processes through which these substrates are made available. The actual factor (or factors) responsible for the initiation of the different gene activities could, therefore, well be related to processes preceding the respiratory chain reactions.

So far, the effects of inhibition of particular steps in the respiratory chain by rotenone and antimycin A indicated that probably only one puff, 81B, is related to a particular reaction of the chain, the dehydrogenation of NADH (see Leenders and Berendes, 1972). It seems, on account of the data presently at hand, unlikely that any of the other puffs has a definite relationship to one of the chain reactions, although their activity may have, by their relationship to reactions preceding and coupled with terminal respiration, a consequence for this chain.

It could be postulated that the puffs produce RNA which carries information for the synthesis of proteins which in some way or another increase the activity of the respiratory metabolism. This support would eventually lead to an adaptation of the respiratory metabolism to the demands of the cell. At the moment at which the balance between the output of the respiratory metabolism and the requirements of the cell is restored, the release of factor(s) initiating the activity of the genome loci would be stopped. This idea finds some support in the observations on the effect of cycloheximide. This antibiotic inhibits puff regression. As such, this effect could be interpreted as being a consequence of the failure of information produced by the puffs to be translated. If so, the puffs should produce information which is translated into a protein (or proteins) which is required for the respiratory metabolism. On the other hand, it cannot be excluded that the products of translation of the RNA produced by the puffs have no relevance with regard to the respiratory metabolism but function as repressor substances at the puff sites in a feedback control system. This possibility was suggested previously for a protein factor which should be active in the repression of ecdysone-induced puff activities (Clever, 1967).

Recently, Ellgaard and Clever (1971) reported changes in the RNA metabolism of salivary glands of *Drosophila melanogaster* as a consequence of a short-term temperature treatment in vitro. They suggested that these changes reflected impairment of RNA processing rather than an actual change in the pattern of RNA species synthesized. Their suggestion was supported by the observation that amino acid incorporation at 24° and 37°C was essentially the same. It cannot be excluded, however, that these results are a consequence of the incubation medium used for these studies, since it is well known that the RNA metabolism in explanted polytene tissues is modified as compared with the in vivo situation (Greenberg, 1969).

In contrast to Ellgaard and Clever's results, the results of a comparison between the behavior of the newly induced puffs and NADH-dehydrogenase activity in the system here described are suggestive of a relationship between the two events. In particular, the effect of cycloheximide which on the one hand inhibits puff regression and on the other hand inhibits the increase in NADH-dehydrogenase activity supports the idea that there exists a definite relationship between the capacity of the respiratory metabolism and the mechanism regulating the activity of the particular puffs. It could be argued, however, that the assay of NADH-dehydrogenase activity used does not discriminate between different NADH-dehydrogenases which may present at various sites within the salivary gland cell and which could have different functions as compared with the enzyme

active in the respiratory chain. In this regard it seems relevant to point to the rapid decline in the NADH-dehydrogenase activity after the puffs have disappeared (Fig. 3), which is indicative of a rapid turnover of the enzyme. The fact that the puffs can be induced repeatedly if the onset of the inductive treatment is repeated within short-time intervals agrees well with the observed rapid decline in NADH-dehydrogenase activity. In terms of puff size, the response of the genome loci to a second or third inductive treatment may not attain the same level as observed after the first induction (Berendes, 1969). It appeared that the maximum response of the puffs to a repeated induction only followed if a certain minimum period of recovery from the first (or previous) induction has passed.

The comparison of the effect of cycloheximide with that of chloramphenicol on both puff regression and NADH-dehydrogenase activity strongly suggests that the increase in enzyme activity results from a *de novo* synthesis of the enzyme (or part of it) by the cytoplasmic translation system. If the *de novo* synthesis of the polypeptide would be governed by information produced in the puffs, the approximate time required for processing of the RNA and translation would be of the order of 30 min under the conditions applied.

Even though the results presented fit the hypothesis that the control of activity of some of the genome loci studied finds its origin in the respiratory metabolism (Leenders and Berendes, 1972), one locus (2-48C) displays in some respects a behavior which is different from that of the other loci. Puff 2-48C regresses after it has been induced in the presence of cycloheximide, whereas the other puffs do not. This indicates that the regression of puff 2-48C can occur independent of the availability of a functioning translation system. Although the behavior of puff 2-48C does not necessarily exclude a possible relationship between the activity of this puff and the respiratory metabolism, it could be that the activity of this locus is related to a different metabolic pathway. The control of activity of puff 2-48C will be the subject of further investigations.

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