PEPTIDES RELATED TO β-LIPOTROPIN WITH OPIOID ACTIVITY

Effects on levels of adenosine 3′:5′-cyclic monophosphate in neuroblastoma × glioma hybrid cells

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1. Introduction

It has been shown that several fragments of the polypeptide β-lipotropin (β-LPH) have affinity for the opioid receptor [1–4]. In addition to the β-LPH-(61–65)-pentapeptide termed methionine-enkephalin (Met[5]-enkephalin) another pentapeptide leucine-enkephalin (Leu[5]-enkephalin) has been isolated [5,6]. The enkephalins and β-LPH, fragments, as well as some analogues of the former, display properties similar to those of morphine in receptor-binding studies [7], in their action on electrically stimulated guinea pig ileum [5] and in the central nervous system, where they cause analgesia [8]. Like morphine these peptides can be specifically antagonized by naloxone in these assay systems. It is also known that the elevation by prostaglandin E_1 (PGE_1) of the intracellular level of adenosine 3′:5′-cyclic monophosphate (cyclic AMP) [9] in neuroblastoma × glioma hybrid cells 108CC15 is inhibited by narcotics [10,11] and enkephalins [12,13]. These cells display many properties characteristic of neurones [14,15]. They constitute a useful model for studying the effects of opioids on the adenylate cyclase system in relation to receptor-binding. Here we report on the levels of cyclic AMP in neuroblastoma × glioma hybrid cells simultaneously exposed to PGE_1 and various β-lipotropin fragments or their analogues. We also compare the changes of cyclic AMP levels with the characteristics of the binding of the same peptides to the opioid receptor in rat brain synaptic membranes.

2. Materials and methods

The synthesis and the analytical data of Met[5]-enkephalin, Leu[5]-enkephalin, β-LPH-(61–69)-nonapeptide, β-LPH-(61–75)-pentadecapeptide and the analogues Phe[1],Met[5]-, Des-Met[5]- and Ala[2],Leu[5]-enkephalin have been reported [7,12,16]. The homogeneous crystalline compounds

H–Tyr–Gly–Gly–OH monohydrate (m.p. 174–176°C; $[\alpha]_{546}^{20}$ + 65.8°C and $[\alpha]_D^{20}$ + 54.4°C (c = 2.0, 20% acetic acid)).

H–Gly–Phe–Leu–OH monohydrate (m.p. 224–225°C; $[\alpha]_{546}^{20}$ − 15.7°C and $[\alpha]_D^{20}$ − 13.9°C (c = 0.2, 80% 2-methoxyethanol)).

Homogeneous H–Arg–Tyr–Gly–Gly–Phe–Leu–OH monoacetate (m.p. 175–178°C; $[\alpha]_{546}^{20}$ + 24°C and $[\alpha]_D^{20}$ + 19.5°C (c = 1.0, 95% acetic acid)).

Amino acid ratios in acid hydrolysate: Arg 1.01 (1), Tyr 0.97 (1), Gly 2.03 (2), Phe 0.99 (1), Leu 1.01 (1) and AP-M digest: Arg 1.04 (1), Tyr 1.00 (1), Gly 2.00 (2), Phe 1.02 (1), Leu 0.98 (1)).

Desamino-Met[5]-enkephalin (m.p. 161–163°C; $[\alpha]_{546}^{20}$ −4.6°C and $[\alpha]_D^{20}$ and −4.2°C (c = 1.0, 95% acetic acid)) and Nle[5]-enkephalin hemihydrate (m.p. 200–202°C; $[\alpha]_{546}^{20}$ + 39.8°C and $[\alpha]_D^{20}$ + 32.5°C (c = 0.9, 95% acetic acid) were synthesized by L.M. and E.W.

Amino acid ratios in acid hydrolysate: Tyr 0.98 (1), Gly 2.00 (2), Phe 0.97 (1), Nle 0.97 (1)).

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PGE_1 was a gift from Dr J. Pike, Upjohn Co., Kalamazoo, Michigan. Morphine · HCl, was purchased from Boehringer, Ingelheim, Germany, levorphanol tartrate and naloxone · HCl were gifts from Hoffmann-La Roche, Grenzach, Germany and Endo Laboratories, Garden City, NY, USA, respectively.

Four days before the experimental incubation 2.4×10^5 viable (exclusion of nigrosin) neuroblastoma × glioma hybrid cells 108CC15 were seeded in plastic Petri dishes (85 mm in diameter) containing 20 ml growth medium [17]. After the experimental incubation (10 min, 37°C) in the presence of PGE_1 and the peptides, the intracellular concentration of cyclic AMP was determined [17]. The opiate receptor-binding assay has been described in detail elsewhere [18].

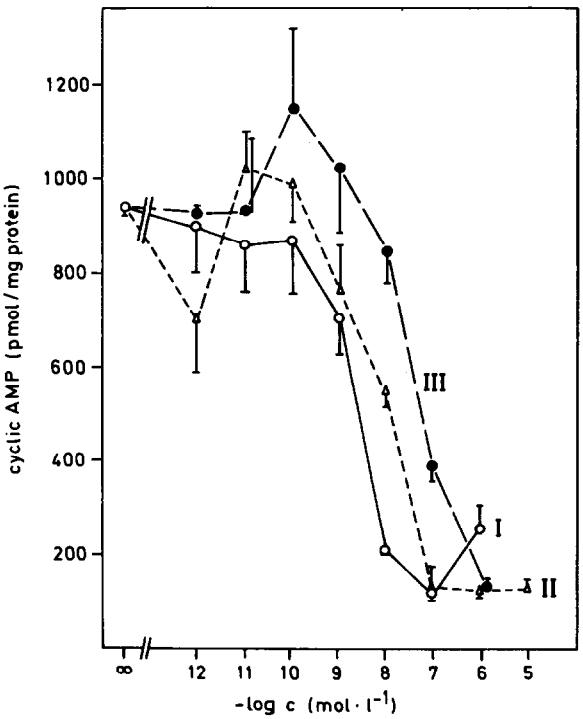

Fig.1. Inhibition by various concentrations of opioid peptides of the increase in the level of cyclic AMP evoked by 0.3 μM PGE_1. Curve (I) Leu⁵-enkephalin, curve (II) β-LPH-(61–69), curve (III) β-LPH-(61–75). 3.0×10^6 viable cells/plate, viability 95%, passage number 14. Mean values ± SD from three parallel incubations.

3. Results

The enkephalins, the β-LPH-(61–69)-nonapeptide and the β-LPH-(61–75)-pentadecapeptide strongly inhibited the increase in the level of cyclic AMP evoked by PGE_1 (fig.1). The parallel concentration–inhibition curves allow quantitative comparison of potency. Met⁵-Enkephalin (curve not shown) and Leu⁵-enkephalin were equipotent (IC_{50}: 1–3 nM). β-LPH-(61–69) (IC_{50}: 10 nM) and β-LPH-(61–75) (IC_{50}: 60 nM) were less potent. Table 1 shows that 10 nM naloxone prevented the inhibitory effect of the peptides. Leu⁵-Enkephalin [19] and β-LPH-(61–69) and β-LPH-(61–75) lower the level of cyclic AMP even in the absence of PGE_1. Structural analogues of the enkephalins were also tested. The results are compared with those of morphine and levorphanol (table 2). With the exception of Nle⁵-enkephalin all analogues were less potent than the enkephalins in attenuating the effect of PGE_1. However, most of them were more potent than levorphanol and morphine. Most interestingly, acylation by an arginyl residue of the amino group of Leu⁵-enkephalin reduced the potency only by one order of magnitude. Strikingly in the rat brain receptor affinity test, the peptides were generally less potent than the opiates. This is obvious if one compares the ratio, IC_{50}/K_D for the

Table 1
Naloxone blocks the inhibitory effect of opioid peptides on the increase in the level of cyclic AMP evoked by PGE_1

Substance added (μM)	Cyclic AMP (pmol/mg protein)
None	38 ± 6
Naloxone (10)	60 ± 11
PGE_1 (0.3)	1850 ± 470
β-LPH-(61–69) (0.1)	21 ± 7
β-LPH-(61–75) (1)	29 ± 2
PGE_1 + naloxone	1920 ± 80
β-LPH-(61–69) + naloxone	41 ± 3
β-LPH-(61–75) + naloxone	38 ± 6
PGE_1 + Leu⁵-enkephalin	241 ± 8
PGE_1 + β-LPH-(61–69)	293 ± 27
PGE_1 + β-LPH-(61–75)	288 ± 18
PGE_1 + β-LPH-(61–69) + naloxone	1890 ± 270
PGE_1 + β-LPH-(61–75) + naloxone	1730 ± 100

The data ± SD are mean values obtained from 3 parallel plate. 2.4×10^6 Viable cells/plate, viability 95%, passage No. 16

Table 2
Comparison of the potency of various opioids in the neuroblastoma × glioma hybrid cell system and in the rat brain synaptic membrane system

Substance	Inhibition of PGE_1-effect on cyclic AMP level in hybrid cells	Binding to receptors in rat brain membranes	Ratio
	IC_{50} (nM)	K_D (nM)	IC_{50}/K_D
Levorphanol	300	3	100
Morphine	500	17	300
Met^5-Enkephalin (H–Tyr–Gly–Gly–Phe–Met–OH)	3	17	0.18
Leu^5-Enkephalin	3	40	0.08
H–Tyr–Ala–Gly–Phe–Leu–OH	50	1200	0.04
H–Arg–Tyr–Gly–Gly–Phe–Leu–OH	30	100	0.3
H–Tyr–Gly–Gly–Phe–Nle–OH	1	30	0.03
H–Phe–Gly–Gly–Phe–Met–OH	2000	100 000	0.02
H–Tyr–Gly–Gly–Phe–OH	10 000	50 000	0.2
β-LPH-(61–69)	10	13	0.77
β-LPH-(61–75)	60	100	0.6

In the hybrid cell system the inhibitory action on the PGE_1-evoked stimulation of cyclic AMP accumulation was studied. In the membrane system the affinity to opioid receptors was measured. Each IC_{50} value was obtained from dose–response curves analogous to those of fig.1.

opiates and the peptides (table 2). A number of peptides were ineffective, i.e., their IC_{50}-values were higher than 10 000 nM. These were: H–Tyr–Gly–Gly–OH (β-LPH-(61–63)). H–Gly–Phe–Leu–OH alone and in combination with β-LPH-(61–63) and desamino-Met^5-enkephalin.

4. Discussion

In the hybrid cells, not only the action of the enkephalins [11] but also that of the longer β-LPH-fragments LPH-(61–69) and LPH-(61–75) is blocked by the opiate-antagonist naloxone. This indicates that the peptides are acting by binding to opioid receptors. Isosteric substitution by norleucine in position 5 of enkephalin rendered a peptide somewhat more active than the enkephalins. Omission from the enkephalins of the C-terminal or of one [7,20] or two N-terminal amino acids or of just the amino group from tyrosine yields oligopeptides devoid of, or almost lacking activity in both assay systems used here. An enkephalin without the phenolic hydroxyl group retains some activity. Interestingly, elongation of the chain at the carboxyl-end by up to 10 amino acids or replacement of Gly in position 2 by L-Ala reduced activity by only one order of magnitude. The tyramine amino group of opiates has been considered essential for biological activity. The fact that the tyrosine of the enkephalins is located at the amino-end, rather than in the internal sequence, appeared to support this notion [21]. Surprisingly, acylation by an arginyl residue of the tyrosine amino group in Leu^5-enkephalin caused but a moderate loss of potency. This demonstrates that maintenance of an amino group or a positive charge in this position is not under all circumstances required for activity. Results from opiate-binding studies led other authors to the same conclusion [22]. This peptide or its Met^6-analogue could arise from a hypothetical variant of β-LPH or from β-LPH, respectively, by tryptic cleavage of the Lys^{59}–Arg^{60} peptide bond. This possibility and the surprising potency of the peptide may suggest its natural occurrence and function. In conclusion, considerable changes in the structure, especially chain-elongation at the carboxyl-end, are tolerated by the enkephalins without extensive loss of biological activity.

For the peptides, the ranking order of potency is similar in the two systems used here and in test systems which analyse analgesic activity [8]. Thus, there is good evidence that the two functional responses, the inhibition of the increase in the level of cyclic AMP and the analgesic action, are related and are elicited via interactions of the peptides with the receptors that can be detected with a binding assay.

However, if the peptides are compared with the opiates, clear discrepancies of potency are found between the systems. In the hybrid cell system the opiates are much less potent than the peptides in inhibiting the increase in the concentration of cyclic AMP caused by PGE_1. In the receptor-binding studies (rat brain synaptic membranes) the situation is the reverse (table 2). It is very unlikely that this large difference is simply due to peptide metabolism during the receptor-binding assay. When tracer amounts of enkephalin were incubated with the membranes carrying the receptors, more than 25% remained as intact enkephalin at the end of the incubation [23]. As a possible alternative is considered that in the two systems, hybrid cells and rat brain membranes, respectively, the peptides and the opiates interact with different receptors.

In case opiates and peptides would act on the same receptor they would be expected to be antagonized by the same antagonist and at a given concentration of an antagonist, a certain agonistic effect should be reduced to the same extent [24]. Indeed, this is observed in the hybrid cell system with naloxone as the antagonist and either Leu^5-enkephalin [12] or morphine as agonists [11,25]. Using the dose–ratio method, Klee and Nirenberg made a similar observation and calculated the K_e (equilibrium binding constant) for naloxone to be 30 nM and 20 nM against Met^5-enkephalin and morphine, respectively. This suggests that naloxone competes with morphine and the enkephalins for the same binding sites. However, an analogous analysis of the synaptic plasma membrane receptors gives different results. Naloxone was allowed to compete with labelled enkephalins or dihydromorphine under strictly identical conditions following the procedure of Terenius and Wahlström [26]. The K_e values of 0.7 nM, 10 nM and 10 nM for naloxone against dihydromorphine, Leu^5-enkephalin and Met^5-enkephalin, respectively, were observed.

Further support for a heterogenous receptor-population in synaptic plasma membranes has been obtained using several competitors against labelled dihydromorphine or labelled Leu^5-enkephalin. Thus, in addition to classical 'morphine sites', there is evidence for additional 'enkephalin' sites [23].

The difference between the hybrid cell and synaptic plasma membrane system are probably real although differences in experimental conditions may play a role. Our tentative hypothesis is that the hybrid cell membrane has only 'enkephalin sites' with high affinity for the peptides but low affinity for opiates. It would be of interest to carry out receptor-binding studies on neuroblastoma × glioma hybrid cells to evaluate this hypothesis.

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References

[1] Ling, N., Burgus, R. and Guillemin, R. (1976) Proc. Natl. Acad. Sci. USA 73, 3942–3946.
[2] Guillemin, R., Ling, N., Burgus, R., Bloom, F. and Segal, D. (1977) Psychoneuroendocrinology in press.
[3] Bradbury, A. F., Smyth, D. G., Snell, C. R., Birdsall, N. J. M. and Hulme, E. C. (1976) Nature 260, 793–795.
[4] Gráf, L., Rońai, A. Z., Bajusz, S., Cseh, G. and Szekely, J. I. (1976) FEBS Lett. 64, 181–184.
[5] Hughes, J. (1975) Brain Res. 88, 295–308.
[6] Hughes, J., Smith, T. W., Kosterlitz, H. W., Fothergill, L. A., Morgan, B. A. and Morris, H. R. (1975) Nature 258, 577–579.
[7] Terenius, L., Wahlström, A., Lindeberg, G., Karlsson, S. and Ragnarsson, U. (1976) Biochem. Biophys. Res. Commun. 71, 175–179.
[8] Büscher, H. H., Hill, R. C., Römer, D., Cardinaux, F., Closse, A., Hauser, D. and Pless, J. (1976) Nature 261, 423–425.

[9] Hamprecht, B. and Schultz, J. (1973) Hoppe-Seyler's Z. physiol. Chem. 354, 1633–1641.
[10] Traber, J., Fischer, K., Latzin, S. and Hamprecht, B. (1975) Nature 253, 120–122.
[11] Sharma, S. K., Nirenberg, M. and Klee, W. A. (1975) Proc. Nat. Acad. Sci. USA 72, 590–594.
[12] Brandt, M., Gullis, R., Fischer, K., Buchen, C., Hamprecht, B., Moroder, L. and Wünsch, E. (1976) Nature 262, 311–312.
[13] Klee, W. A., Lampert, A. and Nireberg, M. (1976) in: Opiates and Opioid Peptides (Kosterlitz, H. W. ed), pp. 153–159, North-Holland, Amsterdam.
[14] Hamprecht, B. (1974) 25th Colloq. Biol. Chem. (Jaenicke, L. ed) pp. 391–423.
[15] Hamprecht, B. (1976) Angew. Chem. Intern. Engl. ed 15, 194–206.
[16] Zieglgänsberger, W., Fry, J. P., Herz, A., Moroder, L. and Wünsch, E. (1976) Brain Res. 115, 160–164.
[17] Traber, J., Fischer, K., Latzin, S. and Hamprecht, B. (1974) Proc. 9th Intern. Congr. Colleg. Int. Neuro-psychopharmacologicum, p. 956–969.
[18] Terenius, L. (1974) Acta Pharmacol. Toxicol. 34, 88–91.
[19] Brandt, M. (1976) Diploma thesis, University of Munich.
[20] Hambrook, J. M., Morgan, B. A., Rance, M. J. and Smith, C. F. C. (1976) Nature 262, 782–783.
[21] Horn, A. S. and Rodgers, J. R. (1976) Nature 260, 795–797.
[22] Chang, J.-K., Fong, B. T. W., Pert, A. and Pert, C. B. (1976) Life Sci. 18, 1473–1482.
[23] Terenius, L. (1977) Psychoneuroendocrinology, in press.
[24] Schild, H. O. (1947) Brit. J. Pharmacol. 2, 189–206.
[25] Klee, W. A. and Nirenberg, M. (1976) Nature 263, 609–612.
[26] Terenius, L. and Wahlström, A. (1976) Europ. J. Pharmacol. 40, 241–248.

SEQUENTIAL INCREASE IN ACTIVITY OF MITOCHONDRIAL ENZYMES DURING RESPIRATORY ADAPTATION OF ANAEROBICALLY-GROWN SYNCHRONOUS YEAST

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1. Introduction

A sequential step-wise increase in activities of a number of mitochondrial enzymes [1–4], as well as in concentrations of cytochromes [5] and of cardiolipin [5,6] were found to take place during the aerobic synchronous growth of yeast-cells. This points to a discontinuous and time-ordered formation, or at least complementation of mitochondria in the cell-cycle of this organism.

Similar step-wise changes in activities of mitochondrial enzymes were also observed in anaerobically-grown yeast during a subsequent synchronous growth under aerobic conditions [2]. In this case, not only the formation of new mitochondria but also a transformation of preformed anaerobic mitochondria (promitochondria) into aerobic mitochondria [7–11] took place. The question whether the latter process did also proceed in a step-wise manner has remained unresolved.

The present paper shows that the activities of cytochrome *c*:O_2 oxidase (EC1.9.3.1.), NADH: cytochrome *c* reductase (EC1.6.2.1), succinate:PMS reductase (EC1.3.99.1) and L-malate dehydrogenase (EC1.1.1.37) increase in a sequential step-wise manner when synchronous anaerobically-grown cells undergo respiratory adaptation under non-growing conditions. This strongly suggests that the transformation of promitochondria into aerobic mitochondria in the yeast cells is a synchronous time-ordered process.

2. Materials and methods

Cells of *Saccharomyces cerevisiae* DT XII were grown under strictly anaerobic conditions for 17 h at 30°C in a semisynthetic medium [12] with 5% glucose as carbon source, supplemented with 0.25% Tween-80, 0.005% ergosterol and 0.4% ethanol.

Synchronous cells were prepared from the asynchronous culture by isopycnic gradient centrifugation procedure [13] as described previously [1,2].

Synchronous non-budding cells representing a population in a middle of the G_1-phase of the cell-cycle were suspended in 50 mM potassium phosphate, pH 5.0, containing 0.25% glucose to a concentration 10^7 cells/ml and incubated under strong aeration at 30°C. Samples were taken in 15 min intervals directly into ice-cold mixture of cycloheximide and chloramphenicol (final concentrations 50 μg/ml and 4 mg/ml, respectively) to prevent protein synthesis. Washed cells were disintegrated with ballotini glass beads [6] and the homogenates analysed for enzyme activities. Spectrophotometric assays were performed to measure the activities of NADH:cytochrome *c* reductase [16], cytochrome oxidase [17], succinate:PMS reductase [18] and malate dehydrogenase using of Boehringer MDH UV Test.

3. Results and discussion

During four hours of respiratory adaptation of the anaerobically-grown synchronous cells in the buffered glucose solution the cells underwent practically no growth and no multiplication; total protein increased by 18–23% and the cell number by 5–10 (fig.1). At the same time, however, the activity of NADH:cytochrome *c* reductase rose as many as 11 times, that of cytochrome oxidase about 10.5 times, of malate dehydrogenase 4.5 times and of succinate:PMS reductase 5 times. As shown in fig.2 the increase of cytochrome oxidase and NADH: cytochrome *c* reductase activities were step-wise at a definite time interval. A minor antimycin A insensitive part of the latter increased at a different time interval than the antimycin A sensitive part (fig.3) which represented about 80% of the total NADH:cytochrome *c* reductase activity.

Malate dehydrogenase activity increased in two steps as it did during aerobic synchronous growth of anaerobically-grown yeast [2], the first minor component having probably corresponded the cytosolic enzyme.

As can be seen from fig.2, the rise in succinate dehydrogenase activity exhibited an oscillatory pattern which was not observed during the aerobic synchronous-growth of both aerobically- and anaerobically-grown *Saccharomyces cerevisiae* [1,2]. This may be an expression of a feed-back control of the synthesis of this dehydrogenase. Oscillation in enzyme activity in the cell-cycle was observed with other organisms and considered as a characteristic of unstable enzymes [14].

The relative time sequence of increase in activity of all the enzymes studied in these experiments with non-growing cells has been found the same as in

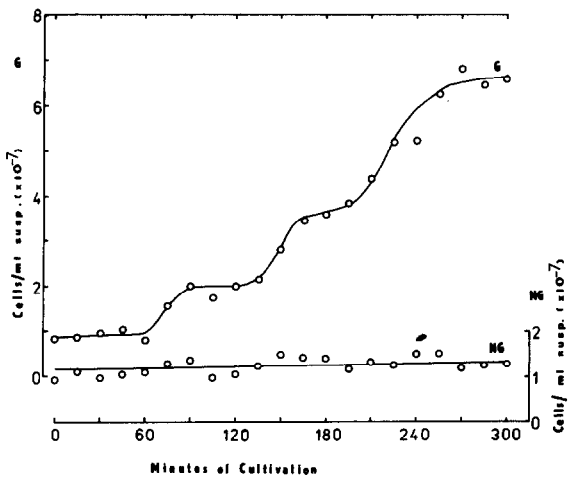

Fig.1. Time-course of the cell-number during the respiratory adaptation of the synchronous anaerobically-grown *Saccharomyces cerevisiae* under non-growing conditions (NG) and separately made growth curve of the same cell-population in the growth medium (G) for the control of cell-synchronicity.

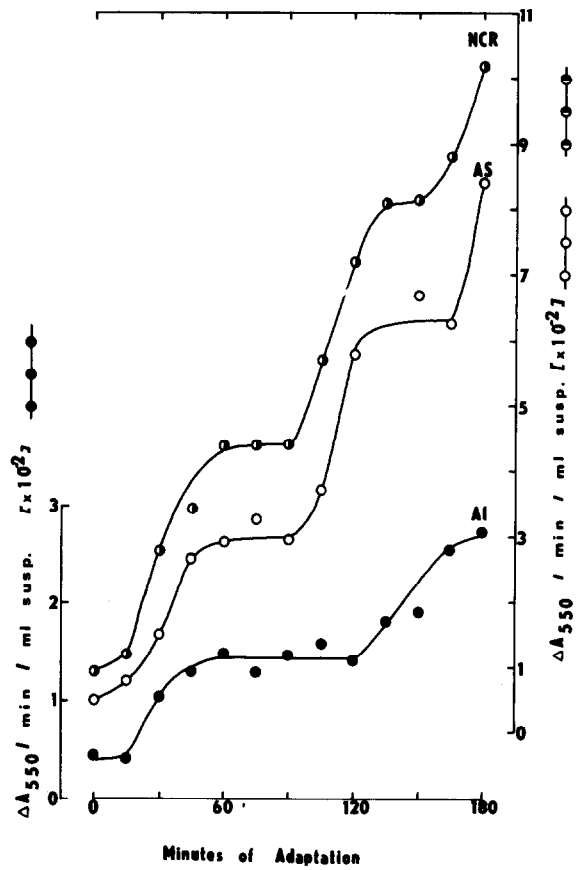

Fig.2. Time-course of the increase in NADH:cytochrome *c* reductase (NCR), cytochrome oxidase (CO), malate dehydrogenase (MDH) and succinate dehydrogenase (SDH) activities during the respiratory adaptation of the synchronous anaerobically-grown yeast-cells under non-growing conditions.

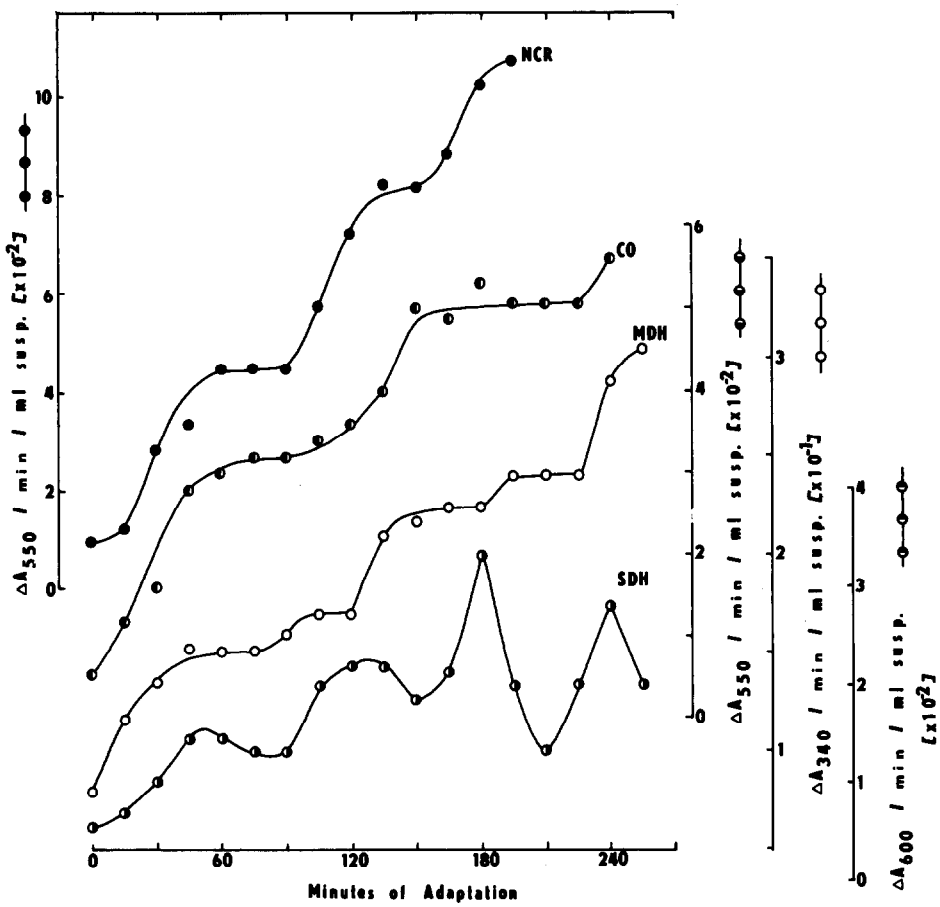

Fig.3. Time-course of the increase in total NADH:cytochrome *c* reductase (NCR), antimycin sensitive (AS) and antimycin insensitive (AI) parts of this enzyme activity during the respiratory adaptation of the synchronous anaerobically-grown yeast-cells under non-growing conditions.

experiments with aerobic synchronously growing cells [1,2].

It might be inferred that the synthesis of the respiratory-chain components in a cell is a sequential time-ordered process independently of whether the components are then being inserted into preformed mitochondria during respiratory adaptation of anaerobically-grown cells or into newly arising mitochondria during cell-growth and multiplication. This would be in accord with the hypothesis of sequentional transcription of genes in the cell-cycle of yeast [15]. It is quite clear, however, that such an interpretation would be too simple to account for a highly complex and coordinate assembly of mitochondria in which transcription of both nuclear and mitochondrial genes, as well as translational and a 'pool-size' controls have been implicated by many studies. Synchronous and synchronized yeast-cells offer excellent opportunities for further exploration of this problem which is tightly connected with the 'cell clock' running even at the stopped cell-growth and division.

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