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Effects of nicotinamide, isonicotinic acid hydrazide, and 3-acetylpyridine on the growth and nicotinamide abenine dinucleo-tide content of L cells

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Abstract

1. Addition of nicotinamide (l0-2M) into the culture medium brings about an increase of the NAD content and the inhibition of the growth of L cells in culture. This rise of NAD brought about by nicotinamide lasts for 2 to 3 days, and thereafter gradually subsiding, it returns to normal level. 2. When L cells are cultured for several days in the same medium without addition of nicotinamide, there occurs a slow-down of mitosis with lapse of cultivation time but it has been found that this is in no way connected with the intracellular content of NAD. 3. By the addition of isonicotinic acid hydrazide (l0-2M) into the culture medium, there can be recognized a decrease of NAD content in L cell and the inhibition of cell growth. 4. In the case when 3-acetylpyridine (l0-2M) is added, a decrease of intracellular content of NAD in L cells and a marked inhibition of the cell growth can be observed. In the groups cultured in the media, containing 3-AP at the concentration of l0-3M or l0-4M can be seen neither inhibition nor acceleration of the cell growth. The oxygen uptake of the cells cultured in the medium containing 3-AP (l0-2M) hardly differs from that of the control group cultured in the medium not containing 3-AP. 5. On the basis of these results discussion has been made on the relation ship between mitosis and NAD content in the cell.

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EFFECTS OF NICOTINAMIDE, ISONICOTINIC ACID HYDRAZIDE, AND 3-ACETYLPYRIDINE ON THE GROWTH AND NICOTINAMIDE ADENINE DINUCLEOTIDE CONTENT OF L CELLS

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As is well known, nicotinamide adenine dinucleotide (NAD) plays an important role as a coenzyme for various intracellular dehydrogenase reactions. An attention is now drawn to the fact that the intracellular content of NAD is markedly decreased in neoplastic tissues as compared with that in normal tissues and that actively proliferating normal embryonic tissues reveal likewise a low value of NAD content when compared with that of corresponding tissues in normal adult[1,2,3,4,5]. Furthermore, it is known that nicotinamide adenine dinucleotide pyrophorylase (NADppase), one of the enzymes that are involved in NAD synthesis, is localized in the nucleus and NAD synthesizing activity of this enzyme is decreased in neoplastic tissue and embryonic tissue same as the NAD contents[6,7]. In his experiment with ameba BRACHET observed a decrease of carbohydrate metabolism in the cells of a denucleated ameba and deduced that the nucleus might be regulating the cell metabolism through the mediation of NAD[8]. Subsequently on the basis of these facts MORTON has assumed that cell division takes place when NAD of cytoplasm decreases to a certain low level, and due to this cell division the NADppase of the nncleus is increased in proportion to a certain amount of cytoplasm. He considers that the difference between embryonic tissue and neoplastic tissue lies in the fact whether or not the amount of NADppase can be increased in harmony with the stimulation of cell division. He is, therefore, of the opinion that in this manner the nucleus regulates not only the cell metabolism but also the cell division through the mediation of the NAD level in cytoplasm[4]. On such an assumption or in view of the important role played by NAD and of the difference in the quantity of NAD in cancer cells and normal cells, cancer chemotherapy is now being attempted with inhibitors or activators for the synthesis of NAD[4,9,10].

On the other hand, the administration of nicotinamide and its relating substances to the mouse markedly increases the NAD content in the liver as roported by KAPLAN *et al*[11]., and also in Japan there are many reports by FUJII and his coworkers dealing with the effects of nicotinamide administration on the cell

division and NAD content[12,10,13,14,15,16,17,18].

With the purpose to scrutinize further the relationship between the growth and the NAD content of cells, observations were carried out about the effects of nicotinamide, isonicotinic acid hydrazide (INH), and 3-acetylpyridine (3-AP) on the mitosis, proliferation and the NAD content of L cells. As the result it has been found that nicotinamide increases the NAD content while INH and 3-AP bring about a decrease in the NAD content, and also it has been recognized that all the three drugs inhibit the growth of the cells. The results of observations are presented in this paper.

MATERIALS AND METHODS

Reagents : Alcohol dehydrogenase (ADH) was extracted by the following method. NAD and 3-acetylpyridine (3-AP) were the products of Sigma Chemical Company and the other reagents were commercial preparations.

Cells : The strain L cells used in the experiment were originally obtained from Tissue Culture Laboratory, Institute for Infectious Diseases, University of Tokyo, about 3 years ago they were stocked in our laboratory by culturing as cell sheet on one side of the rectangular culture bottle B (the products of Ikemoto Rika Kogyo Co., Ltd.) and by continuing subculture twice a week. For the subculture the cells are dislodged from the wall of the bottle with rubber cleaner and they are dispersed in the culture medium by gentle pipetting.

Culture medium : For the experimental groups the medium used was composed of 70 parts of saline D[19] containing 0.08 % yeast extract and 0.4 % lactalbumin hydrolysate, 20 parts of bovine serum, and 10 parts of the aqueous solution of either nicotinamide or INH at an appropriate concentration. For the control group, the same medium substituted with an equal volume of distilled water in place of the aqueous solution of nicotinamide or INH. As there is hardly any difference in the growth of L cells with 10 % bovine serum in place of 20 % under the present experimental condition, the medium composed of 80 parts of saline D, 10 parts of bovine serum, and 10 parts of 3-AP aqueous solution was used in the experiment for the administration of 3-AP. For the control, the medium was consisted of the same with exception that 3-AP aqueous solution was replaced by an equal volume of distilled water.

Culture methods : Those actively proliferating L cells are dislodged from the wall of culture vessel by rubber cleaner and the suspension is made by gentle pipetting the cells into the medium. The culture medium suspended with an appropriate number of L cells is inoculated into TD-15 flasks or rectangular culture bottles B according to the purpose of the exepriment and the static culture is carried out in an electric incubator at 37°C. Two ml of the suspension medium

is put in a TD-15 flask and 10 ml of it in a rectangular bottle B. In this instance, EDTA treatment is not done nor trypsin digestion resorted to. Since a large number of the cells is required for the assay of NAD, the inoculum size is increased to 200,000 to 300,000/ml at some sacrifice of proliferation and the inoculation is conducted in the rectangular bottles B.

Determination of mitotic rates : For the determination of mitotic rates colchicine solution in the volume 1/10 of the culture medium is added to the medium 10 hours prior to harvesting. Colchicine is dissolved in 0.85% sodium chloride solution in such an amount as to make its final concentration 1/40,000 w/v when added to the culture medium[20]. At harvesting, the cells are well suspended in the medium by pipetting, and this is subjected to centrifugation at 2,000 r. p. m. for 15 min and the supernatant is removed by decantation. The cells are resuspended in a small amount of supernatant remaining in the centrifuge tube, and this suspension is smeared on a slide glass. After methanol fixation and Giemsa staining, about 3,000 cells are counted under a microscope, and the number of those cells resting in mitotic stage out of 1,000 cells is made to represent the mitotic rates.

Morphological observation : Small pieces of coverglass are placed in TD-15 flasks and the medium containing L cell suspension is gently placed on each piece of coverglass, and the incubation is carried out under the same conditions as in other culture. The cells proliferated on the coverglass are fixed with methanol, stained with Giemsa solution and embedded in balsam for microscopic observation.

Extraction of alcohol dehydrogenase : The baker's dry yeast, obtained from the Oriental Yeast Co., Ltd., is used as the source of enzyme. This is first grounded in a homoblender (Sakuma Mfg. Co.) while being cooled and strained through 60-mesh strainer. By this procedure a powder form of yeast cell and/or cell debris is obtained for extraction of the enzyme. OKUNUKI and SEKUZU's method[21] based on that of RACKER[22] is employed for the extraction of alcohol dehydrogenase with slight modification according to the type of apparatus. The alcohol dehydrogenase thus prepared is dispersed in 50% saturated ammonium sulfate solution and is stored in a deep freezer at −20°C.

Extraction of NAD : CIOTTI and KAPLAN's method[23] is slightly modified as to be adaptable to the cells in culture. As it is not so easy to obtain a large number of cells in culture in the same state, it is necassary to find out the minimum number of cells that would give a reliable value of NAD content. By the determination of NAD content in the extracts from various number of cells it has been found that the minimum of 10,000,000 cells is necessary for the extraction of NAD to determine the reliable value. Therefore in the following experiments the number of the cells slightly larger than this minimum is used. At first, the

samples of the cells suspended in the medium by gentle pipetting are used for counting cells on one hand and for the assay of NAD on the other. Those cells used for the NAD assay are subjected to centrifugation at 5,500 g for 10 min and the supernatant is separated by decantation and further the liquid on the tube wall is wiped off with filter paper and the weight of the precipitate is quickly estimated. This weight is taken as the fresh weight. The precipitate is resuspended in 1 ml of cold 5 % trichloracetic acid (TCA) and it is removed to a glass homogenizer avoiding its adhesion to the wall. To the centrifuge tube 0.5 ml TCA is added and all the remnant is removed into the glass homogenizer. This is homogenized for 90 sec while cooling and it is immediately centrifuged at 0°C, 24,000 g for 20 min, and the supernatant is removed to the short test tube as completely as possible. TCA is extracted from the supernatant solution five times with 1.5 ml of ether each time. As the volume of the sample hardly changes throughout this extraction, this is used for the NAD assay.

Assay of NAD: 1.7 ml of 0.15 M sodium pyrophosphate, 0.1 ml of 5 M ethyl alcohol, 0.1 ml of 0.1 M semicarbazide and 1.0 ml of NAD extract are mixed in a silica cuvette and the absorbance is read at the wave length of 340 mμ (Reading I). To this 0.1 ml (80 r) of ADH solution is added and the absorbance is read at 340 mμ. This is left standing for a short while to make it certain that there occurs no change in the absorbance (Reading II). Further, 0.1 ml of ADH solution is added to this and the absorbance is read at the same wave length (Reading III). From these, when we consider the increase in the absorbance due to the reduction of the NAD contained in 1 ml of the extract as R, we get; $R \rightleftharpoons R_2 - R_1 - (R_3 - R_2)$.

The quantity of NAD in 1.5 ml of the extract can be calculated from this R by using millimolar extinction coefficient for NAD (6.2). As for the ADH solution, the original solution stocked in the deep freezer is diluted 1 : 10 with distilled water each time and this diluted solution is always confirmed to have a sufficient activity before its use. In this instance, as there is no effect on the value obtained even without addition of semicarbazide hydrochloride, after using this only several times, sodium pyrophosphate (0.15 M) is substituted in its place. The final pH of the solution assayed is over 9.5 and within 10, showing approximately a uniform value. For the purpose of the correct comparision between the test group and the control in most cases both are extracted and assayed simultaneously by identical method. In order to minimize the length of procedure time before the extraction, one sample each of the test group and the control is taken as a set of the experiment, avoiding the assays of many samples at a time.

Extraction of NADH: For the extraction of NADH, JEDEIKIN and WEINHOUSE's method[1] is employed for the cultured cells. The number of L cells to be

used for the assay is the same as in the case with NAD, and since NADH readily decomposes, the cells after dislodging from the cluture vessel wall are immediately cooled to 0°C and transferred to the centrifuge tubes, centrifuged at 0°C, 5,500 g for 10 min, decanted to remove supernatant and the liquid on the wall of centrifuge tube is wiped off with filter paper. Prior to the homogenization, the combination of 1 ml of phosphate buffer (0.05 M, pH 8.7) and 0.5 ml of distilled water placed in a glass homogenizer is immersed in the boiling bath for more than 5 min. The precipitate mentioned above is removed as quickly and completely as possible into the homogenizer and immersed in the boiling bath for 60 sec. Immediately after this it is homogenized for 30 sec and centrifuged at 0°C, 24,000 g, for 20 min and then the NADH assay is done with the supernatant thus prepared.

Assay of NADH: In a silica cuvette 1.5 ml of 0.1 M phosphate buffer (pH 7.0), 0.1 ml of 10% acetaldehyde solution, 0.3 ml of distilled water, and 1.0 ml of NADH extract are mixed well, and absorbance is read at the wave length of 340 mμ (Reading I). Further, 0.1 ml of ADH solution is added to this and its absorbance is measured (Reading II). By adding again 0.1 ml of ADH solution, the absorbance is read at the same wave length (Reading III). The decrease, R, of the absorbance due to the oxidation of the NADH contained in 1 ml of the extract would be: $R \fallingdotseq R_1 - R_2 + (R_3 - R_2)$

As in the case with NAD, the calculation of NADH is done from this value in an identical manner. As for the counting of L cells, it is taken from those cells cultured in another vessel under the identical conditions. In this manner the assay of NADH is conducted at various conditions of the culture, but NADH is hardly detectable, and even in the case where it is detected, it is in a very small amount as compared with that of NAD, being always less than 1/10 of the latter. Therefore, what is represented as the NAD content in the following is the estimated value of oxidized NAD as it is.

Assay of INH analog and 3-acetylpyridine analog (APNAD) of NAD: As for the INH analog of NAD, it is measured at the time of NAD assay in the group inoculated with INH but before the addition of ADH at the wave length of 385 mμ, which is specific to this analog[23], and the presence of INH analog is presumed by the increase in the absorption at the wave length but its absolute quantity is not measured.

The estimation of APNAD is done with KAPLAN's method[23]. In this instance, the calculation is made with millimolar extinction coefficient at the absorption maximum, 365 mμ, of APNADH as 9.1.

RESULTS

1. *Effect of nicotinamide on the growth of L cells*

After inoculating nicotinamide at the concentration of 10^{-2} M into the culture medium, observations are conducted to see how it affects the growth of L cells. For the culture TD-15 flasks are used. As illustrated in Fig. 1, the result reveals that in the group treated with nicotinamide the cell proliferation is clearly sup-

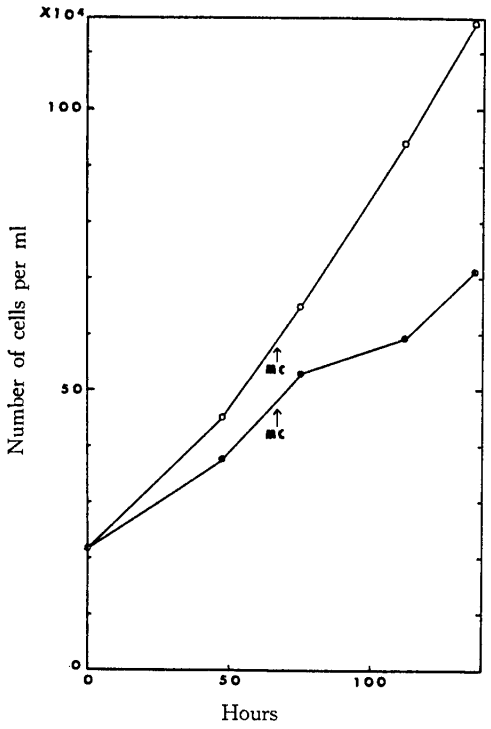

Fig. 1. Effect of nicotinamide on the growth of L cells. ·——·, the growth of L cells cultivated in the medium containing nicotinamide (10^{-2}M); o——o, control.

pressed. In similar experiments conducted several times, although there can be recognized some differrences in the degree of the inhibitory effect, the inhibition can be observed each time. In addition, there can be seen hardly any differences in the medium pH and the cell structure between the test group and the control.

2. *Effect of nicotinamide on the mitotic rates of L cells*

Influence of nicotinamide on the mitotic rates of L cells can naturally be anticipated from the foregoing experiments, but this test is conducted with the aim to determine numerically the extent of its inhibitory action on the cell proliferation. As already described in the foregoing, the number of the resting cells in mitotic stage out of 1,000 cells whose mitosis is stopped at metaphase by colchi-

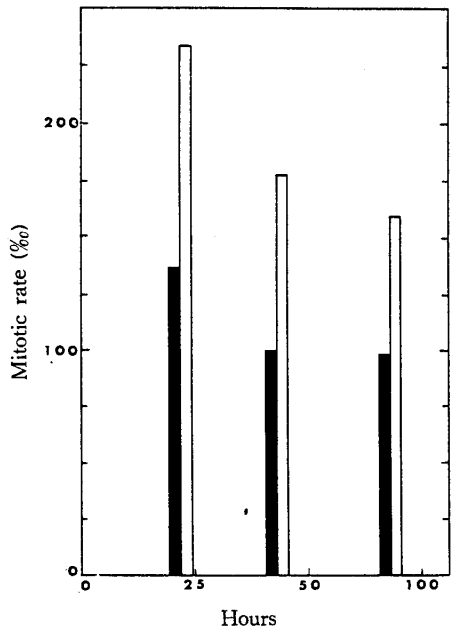

Fig. 2. Effect of nicotinamide on the mitotic rates of L cells.
Shaded bars show the mitotic rate of the cells cultivated in the medium containing nicotinamide (10^{-2}M). Open bars show the control. Mitotic rates determination with colchicine method is described in the text.

cine is represented as the mitotic rate. The result is shown in Fig. 2. It demonstrates more distinctly the inhibition of cell proliferation by the administration of nicotinamide than in Experiment 1. In the similar condition, when cultivation is conducted for a longer period of time with changing of the medium during the incubation, the mitotic rate decreases more and more with lapse of cultivation time, and the difference in the mitotic rate between the test group and the control becomes smaller. The fact that mitotic rates of both the test group and the control slow down gradually with lapse of cultivation time means that the cell proliferation is not undergoing logarithmically, and this seems to be due to the fact that the conditions of culture and the state of cultured cells have not been in optimal conditions.

3. *Comparison of the NAD contents and the mitotic rates between the group treated with nicotinamide and the control*

The nicotinamide solution inoculated was at the concentration of 10^{-2} M, and the rectangular culture bottles B served as the culture vessels. The results, demonstrated in Table 1, show that NAD in the group treated with nicotinamide is clearly increased as compared with the control and there can be seen a fall in the mitotic rate, too. In comparing the NAD contents and mitotic rates

Table 1. Effects of nicotinamide on the NAD content and mitotic rates of L cells. Experimental culture medium contains nicotinamide (10^{-2}M) in the control culture medium.

	Cultivation time (hrs)	Number of cells (x10^6)	NAD content per gram of fresh cells (μg)	NAD content per cell ($\mu\mu$g)	Mitotic rates (‰)
Exp.	24	17.1	196	0.97	116
Cont.	24	20.7	162	0.64	174
Exp.	48	22.7	203	0.87	84
Cont.	48	24.9	135	0.58	163
Exp.	25	14.5	178	0.99	66
Cont.	25	15.0	110	0.55	88

among the test groups or among the control groups, there can be found not necessarily any mutual relationships.

4. *Relationship between the NAD content and the growth of L cells cultivated in the control medium*

As can be observed in Experiments 1 and 2, under such culture conditions the cell proliferation does not necessarily proceed logarithmically. Even when

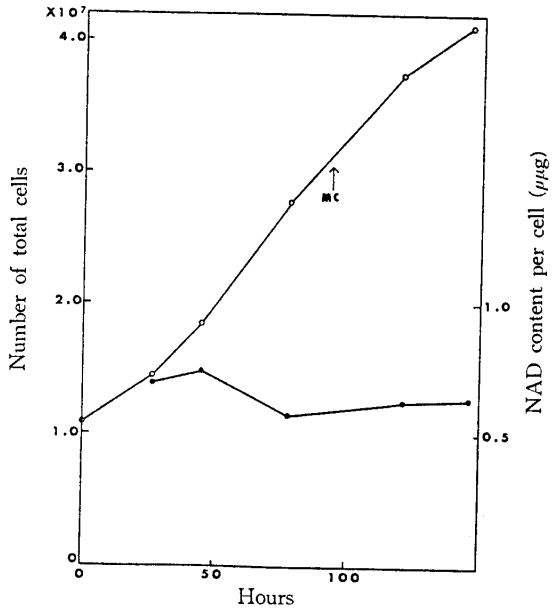

Fig. 3. Relationship between the NAD content and growth of L cells cultivated in the control medium.

o——o, the growth of L cells cultivated in the control medium; ·——·, NAD content of the cell.

the medium is changed on about the third day, no marked acceleration in the proliferation is observable, but when these cells are dispersed in a new culture medium and the inoculum size is decreased, the cells proliferate actively. From these facts it appears that interaction among the cells is considerably associated with the inhibition of cell proliferation occurring along with the lapse of cultivation time. It is obvious that there occurs an inhibition of cell proliferation with lapse of cultivation time even without inoculation of nicotinamide, and with the aim to determine how the NAD content in the cell behaves at this instance, L cells are cultured in the medium used for the control group to see

Table 2. Relationship between the growth and NAD content of L cells cultivated in the control medium.

Cultivation time (hr)	Fresh weight (g)	Number of cells ($\times 10^6$)	NAD content per gram of fresh cells (μg)	NAD ontent per cell ($\mu\mu$g)	Estimated mitotic rates
0		10.9			
26	0.047	14.4	215	0.695	128
45	0.071	18.4	195	0.740	139
78	0.091	27.8	172	0.570	94
*					
121	0.135	37.5	173	0.624	51
146	0.139	41.1	188	0.637	

* Medium was changed after 94 hours of the inoculation.

whether or not the intracellular content of NAD undergoes any change along with lapse of cultivation time. The results, as shown in Fig. 3 and Table 2, reveal that NAD is decreased on about the third day but on the whole there can be recognized no mutual relationship between the duration of culture and the intracellular NAD content. The number represented as estimated mitotic rates in Table 2 is the value deduced from the proliferation curve, and the mitotic rates are deduced from that in 1,000 cells that are supposed to have stopped their mitosis at metaphase during 10 hours, the same as in the group treated with colchicine.

5. *Hourly changes of the NAD content in the L cells incubated in the medium containing nicotinamide*

L cells are incubated under identical conditions as in the test group of Experiment 3 in order to see how the intracellular NAD changes.

KAPLAN *et al.*[11] have observed that, when nicotinamide is administered to the mouse, the NAD content in the liver reaches its peak level 8 to 12 hours after the injection and it returns practically to the normal about 24 hours later. As L cells in culture are the aggregate of the cells of the same strain, the meta-

bolism of nicotinamide or NAD is supposed to depend on the control mechanism of each cell. In addition, as it is possible to consider that there is always a large amount of nicotinamide in the medium and its terminal metabolites are excreted into the medium, it is only natural to assume that there is difference in the change of the intracellular NAD contents of the mouse liver and that of the NAD in a single culture cell when nicotinamide is administered. The result of the experiment, as shown in Fig. 4, reveals that the NAD content per cell reaches its maximum level for 2 to 3 days after the inoculation of nicotinamide and thereafter gradually decreases to the normal level. In this experiment a medium

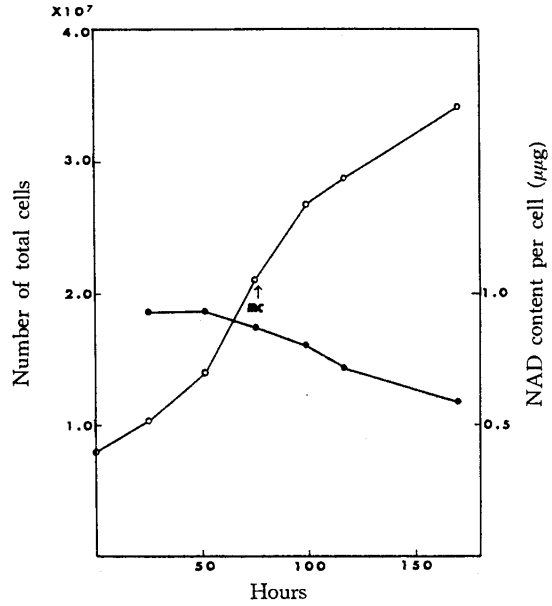

Fig. 4. Hourly changes in the NAD content in the L cells cultivated in the medium containing nicotinamide.

o——o, the growth of L cells cultivated in the medium containing nicotinamide (10^{-2}M); •——•, NAD content of the L cell.

exchange was done at the 76th hour of the incubation with a new medium containing identically the same quantity of nicotinamide but the NAD content did not show any tendency to increase again. In a separate experiment the control medium cultivating cells was exchanged by the medium containing nicotinamide and when the NAD content was estimated at the 12th hour after this exchange, the NAD value did not differ appreciably from the maximum in this experiment. However, what trend observable in this experiment is the fact that the NAD content once increased by nicotinamide administration returns ultimately to the normal level, and that this decrease of the NAD content to the

normal level does not signify an increase in the rate of cell proliferation which was initially suppressed by the nicotinamide administration. In this experiment, as a large number of cells is required for the assay of NAD, the proliferation rate was somewhat sacrificed, as mentioned already.

6. *Effect of INH on the growth and the NAD content of L cells*

After inoculating 10^{-2} M INH in the culture medium the proliferation of L cells and the intracellular NAD content were observed. The result, as illustrated in Table 3, clearly demonstrates a fall in the NAD content per cell or per gram

Table 3. Effects of isonicotinic acid hydrazide on the growth and NAD content of L cells. Experimental culture medium contains isonicotinic acid hydrazide (10^{-2}M) in the control culture medium.

Culture No.		Inoculum size (x10^6)	Cultivation time (hr)	Number of total cells (x10^6)	NAD content per gram of fresh cells (μg)	NAD content per cell ($\mu\mu$g)
1	Exp.	9.0	45	12.2	68	0.313
	Cont.	9.0	45	19.5	219	0.711
2	Exp.	14.1	46	17.8	91	0.321
	Cont.	14.1	46	19.8	173	0.750
3	Exp.	6.0	45	10.3	101	0.420
	Cont.	6.0	45	13.5	145	0.530
	Exp.	6.0	96	21.5	121	0.407
	Cont.	6.0	96	25.4	156	0.584
	Exp.	6.0	116	19.8	81	0.290
	Cont.	6.0	116	30.7	165	0.630
4	Exp.	9.2	46	16.2	115	0.380
	Cont.	9.2	46	20.5	174	0.560
	Exp.	9.2	96	18.5	102	0.410
	Cont.	9.2	96	24.1	140	0.600

of fresh cells and the inhibition of cell proliferation, but the inhibition being not complete, the cells continue to proliferate. Moreover, the extent of the inhibitory action on the cell proliferation in this instance is somewhat higher than in the case where 10^{-2} M nicotinamide is inoculated. ZATMAN *et al.*[28,29] pointed out the formation of INH analog of NAD, though the author did not analyze the formation of INH analog of NAD qualitatively, he observed an increase in absorbance corresponding to the maximum absorption wave length of INH analog, 385 mμ.

7. *Effect of 3-acetylpyridine on the growth of L cells*

In this experiment L cells were cultured in TD-15 flasks containing 10^{-2} M, 10^{-3}M, or 10^{-4}M 3-acetylpyridine (3-AP) to observe the effect of this drug on

the cell proliferation. The control group was cultured in the medium containing the equal amount of distilled water in place of 3-AP. As the result in the cells incubated in the medium containing 3-AP at the concentration of 10^{-2}M a marked inhibition of the proliferation can be recognized as compared with the control (Fig. 5). In those cells incubated in the medium centaining 3-AP at the concent-

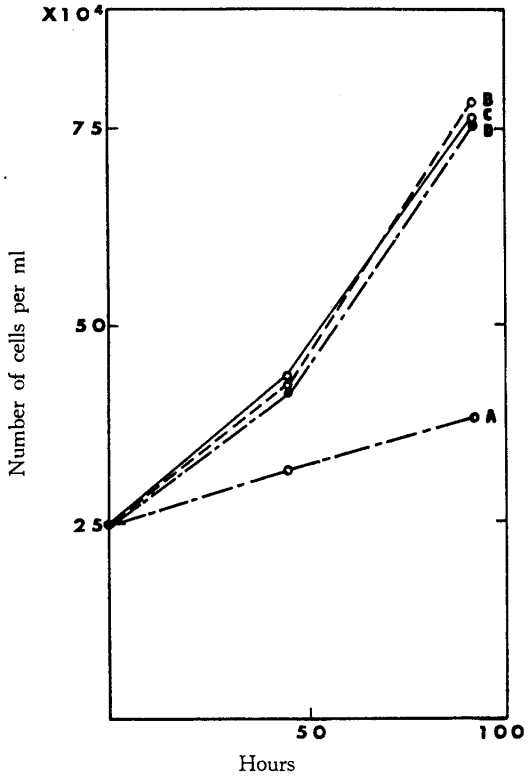

Fig. 5. Effect of 3-AP on the growth of L cells.
A, B, and C show the growth of L cells cultivated in the medium containing 3-AP in the concentrations of 10^{-2}M, 10^{-3}M, and 10^{-4}M, respectively. D shows the control.

ration of 10^{-3}M or 10^{-4}M neither inhibition nor acceleration of the cell growth can be recognized, the same as those cultivated in the control medium. For the purpose to see whether or not such an inhibitory action of 3-AP at the concentration of 10^{-2}M is reversible, the cells were incubated in the medium containing 3-AP at the same concentration and the medium was exchanged one or two days later with the medium not containing 3-AP just as in the control and the culture was continued. As the result it was observed that theoe cells first cultured in the medium containing 3-AP and exchanged with the control medium regained their

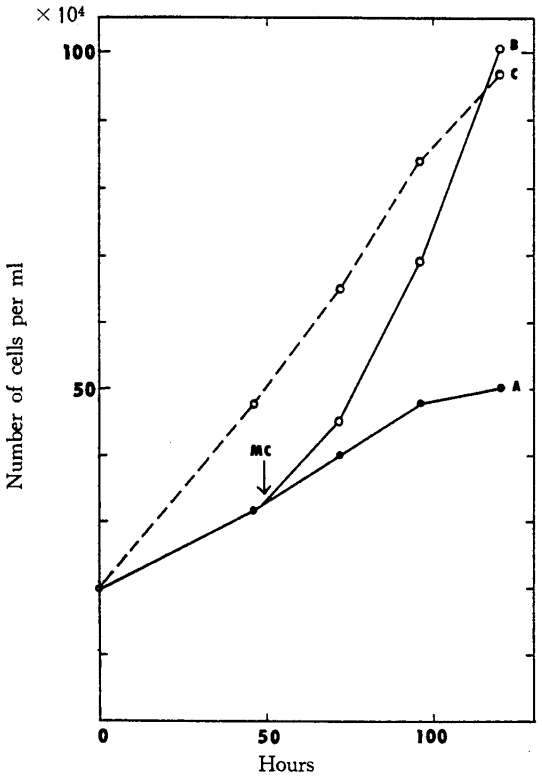

Fig. 6. Effect of 3-AP on the growth of L cells.
A, ·——·, shows the growth of L cells cultivated in the medium containing 3-AP (10^{-2}M). B, ○——○, shows the growth of L cells cultivated at first in the medium containing 3-AP, and then the medium was exchanged 48 hours after inoculation with the control medium and cultivation was continued. C, ○·····○, shows the control.

proliferative power and did multiply actively (Fig. 6). In this experiment the pH of the medium containing 3-AP initially shows hardly any change, but with several days of culture it is found that the pH of the control is somewhat lower than the test group. This is thought to be due to the fact that the proliferation of the control group is greater and hence the number of the cells is increased. A similar tendency can be observed in the groups incubated in the medium containing nicotinamide or INH.

8. *Effect of 3-AP on the NAD content of L cell*

In this experiment 3-AP was inoculated into the medium at the concentration of 10^{-2}M, at which the cell growth was markedly inhibited in the foregoing experiments. As a large number of cells is required for the assay of APNAD, inoculum size was increased even at the expense of some loss in the rate of cell

Table 4. Effects of 3-acetylpyridine on the growth and NAD content of L cells. Experimental culture medium contains 3-acetylpyridine (10^{-2}M) in the control culture medium.

Culture No.		Inoculum size (x10^6)	Cultivation time (hr)	Number of cells (x10^6)	NAD content		APNAD content	
					per cell ($\mu\mu$g)	per g. f. c. * (μg)	per cell ($\mu\mu$g)	per g. f. w. (μg)
1	Exp.	10.3	45	12.7	0.203	50	0.146	36
	Cont.	10.3	45	19.9	0.529	145	—	—
	Exp.	10.3	95	16.6	0.242	59	0.045	11
	Cont.	10.3	95	27.8	0.520	148	—	—
2	Exp.	18.5	24	19.7	0.381	92	0.053	13
	Cont.	18.5	24	27.2	0.547	175	—	—
3	Exp.	12.0	42	19.2	0.278	80	0.092	26
	Cont.	12.0	42	22.4	0.750	201	—	—
4	Exp.	9.4	43	15.3	0.424	105	0.016	4
	Cont.	9.4	43	19.4	0.770	185	—	—
	Exp.	9.4	71	22.9	0.294	96	0.000	0
	Cont.	9.4	71	29.1	0.550	174	—	—

* g. f. c.: abbreviation of gram of fresh cells

proliferation. The results are shown in Table 4. After 3-AP inoculation there were observed a marked inhibition of cell growth as in the preceding experiment and further a clear-cut decrease in the intracellular NAD content, as well as the formation of 3-acetylpyridine analog (APNAD) of NAD. The decrease in NAD brought about by 3-AP is somewhat larger than that by INH. However, since the culture with these two different drugs were not conducted simultaneously under same condition, it is impossible to make correct comparison.

9. *Respiration of the L cells cultured in the medium containing* 3-*AP*

In order to find out whether the decrease of NAD content in a cell would result in the change of oxygen consumption in the cell, the experiment was conducted under identical conditions as in Experiment 8, and the oxygen uptake by the cell was measured with a Warburg manometer. In the main chamber was placed 1.8 ml of glucose free Hanks solution suspended with the cells to be tested; in the side chamber 0.2 ml of 0.2 M glucose dissolved in glucose free Hanks solution, and in the well 0.2 ml of 20% KOH. At the time of measurement the content of the side chamber was transferred into the main chamber and the measurements were taken at 37°C. For the purpose to measure the endogeneous oxygen uptake, glucose free Hanks solution was placed in the side chamber in place of the glucose solution. As shown in Fig. 7, there could be

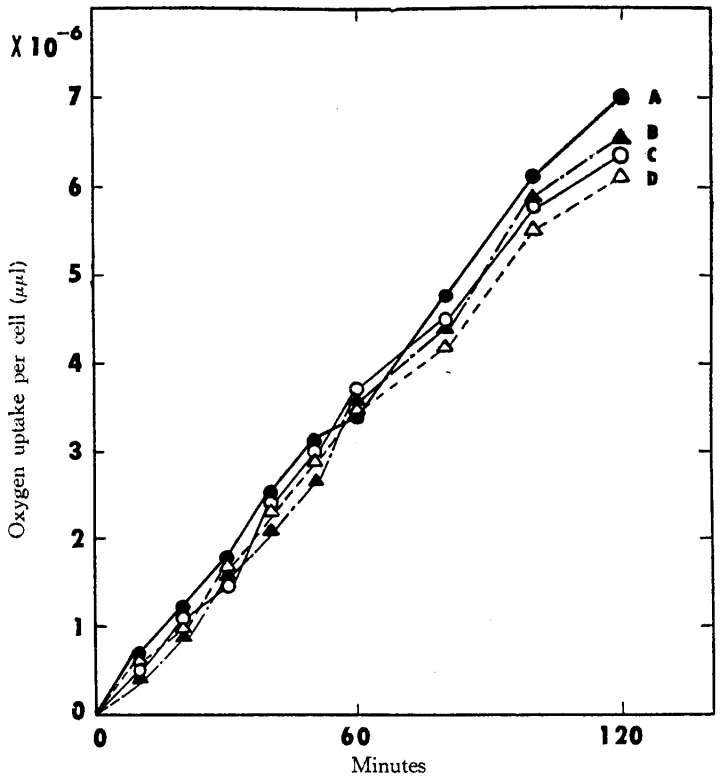

Fig. 7. Oxygen uptake of L cells cultivated in the medium containing 3-AP and control medium, respectively.

A and B show the oxygen uptake of the cells, cultivated in the medium containing 3-acetylpyridine, in glucose free Hanks solution added 0.02M glucose and glucose free Hanks solution, respectively. C and D show the control in each case, respectively.

recognized no significant difference in the oxygen uptake between the cells cultured in the medium containing 3-AP and the control. Another experiment was also conducted with 0.2 ml of the combination of 0.1 M sodium malonate and 0.1 M sodium pyruvate, dissolved in glucose free Hanks solution, placed in the side chamber in place of 0.2 M glucose solution of the previous experiment. They had no marked effects on the oxygen uptake of these cells.

10. *Morphological observation of L cells cultivated in the medium containing nicotinamide, INH, or 3-AP of 10^{-2}M concentration*

In the microscopic observations of the cells grown on the small pieces of coverglass placed in TD-15 flasks, there could be recognized hardly any morphological differences among those cells in the medium containing nicotinamide,

INH, 3-AP and control groups. When the incubation is continued without medium change, however, the cells in the control become detached from the culture vessel wall sooner than the cells in the nicotinamide, INH, and 3-AP treated groups. With this an acceleration in the increase of cell population and an accumulation of metabolites seem to be associated, but precise study has not been done.

DISCUSSION

As already mentioned in the introductory, concerning the relationship between NAD content and mitoses, MORTON[4] assumed that, when the fall in the concentration of NAD in cytoplasm reaches a certain critical low level, mitosis is elicited by virtue of feed-back mechanism, on the basis of the findings that the activity of NADppase and NAD content in various cancer tissues and the tissues of immature mice are lower than those in normal tissues and that NADppase, which is involved in NAD synthesis, is localized in the nucleus[4]. Similarly, FUJII and co-workers have thought that, when NAD decreases to a certain critical point, cell division is induced and they have tried some experiments to validate this assumption.[10,15]

What differs in the idea of these two schools is in the point that MORTON *et al.* consider the rate limiting factor of the fluctuations in NAD content of the cell to be NADppase whereas FUJII *et al.* consider it to be nicotinamide adenine dinucleotidase (NADase) and the latter have attempted to verify their idea. First of all, let us consider the basic fact that has induced such an assumption. A cell is not only concerned with maintenance of its life but also with its proliferation and it has a special function to perform. On the other hand, NAD plays an important role as a co-enzyme in various dehydrogenase reactions, and this is directly connected to the energy production in a cell. For the maintenance of life, growth, mitosis, and differentiated function of a cell, NAD is thought to be essential. It is needless to say that separate NAD does not perform these different functions in a cell but it operates as an entity in a well-coordinated cell activity. Even in normal adult animals NAD content differs considerably according to different organs.[3,14] This is not easily explained simply by the difference in its vegetative activity, but it seems that various factors such as difference in the proportion of cell components, difference in cell metabolism and functional activity in an organ, are also much concerned. It is known that in the liver of embryonic rats and newborn rats NAD content is low but it increases rapidly after birth, reaching the same level as in adult rats in about one week.[5] It might be assumed that in response to this phenomenon mitosis decreases rapidly after birth, but it would be more rational to think that the change in NAD occurs due to the alteration of cell metabolism and/or functional activity. In cancer

tissue vegetative activity is high but the functional activity is almost or completely nil. In the normal tissues of adult animals vegetative activity is generally low but functional activity is high. This difference in the NAD content between the two is in response to the difference in the activity and the difference in metabolism, and this can be considered as an attendant phenomenon on the common basis of cancerization. It seems premature to connect mitosis with the decrease in NAD on the basis of these differences alone. However, the present state of knowledge where the chemical regulatory mechanism of cell division is not yet clarified, it may be significant to carry on the studies with such a working hypothesis. By increasing NAD content after administration of nicotinamide FUJII and his coworkers have observed the decrease in mitosis. Recently they have also observed an increase of NADase activity and a decrease of the NAD content in the sea-urchin egg after fertilization.[16] As this phenomenon is the one under a special state of fertilization, it cannot be applied at once to mitosis in general, nonetheless, it is a step forward to the solution of the problem. On the other hand, it is thought that the intracellular NAD content is maintained at a certain standard level by the balance between the rate of synthesis and that of decomposition. As for the enzymes that decompose NAD, NADase and nicotinamide adenine dinucleotide pyrophosphatase are known.[25,26] The former, NADase, is an enzyme that attacks at the nicotinamide riboside linkage of NAD and thus it hydrolyzes NAD, but in the presence of nicotinamide or related compounds, acting as a transglycosidase, it catalyzes the exchange reaction between nicotinamide moiety of NAD and its pyridine compound. It is known that, should the pyridine compound proves to be nicotinamide, it results in the prevention of decomposition of NAD and brings about an increase of NAD, and if it is a pyridine compound other than nicotinamide, it elicits the formation of NAD analog of that pyridine compound. As for the pyridine compounds that form NAD analog through this exchange reaction, isonicotinamide, isonicotinic acid hydrazide (INH), marsilid, 3-acetylpyridine (3-AP)[27], ethylnicotinate, and others are known[9]. In the present experiments the author has observed the effects of nicotinamide, INH, and 3-AP on the growth and the NAD content of L cells. The reason for selecting L cells, a kind of tissue culture cells, lies in the fact that it is easy to know the cell number, and also the rate of proliferation, mitosis as well as the average NAD content per cell. In addition, it is relatively easy to fix the conditions of the test groups and the control with least necessity for taking their functional activity into consideration. An example of the results obtained in these experiments may be summarized as in Table 5. Even in the experiment with L cells cultivated in the medium containing nicotinamide, results similar to those of FUJII were obtained, namely, NAD content in the cell increases and mitosis decreases. In this instance, it seems reasonable to assume that the

Table 5. Summerized data of the present experiments: the growth rates and NAD content of L cells cultured in the medium containing nicotinamide, isonicotinic acid hydrazide and 3-acetylpyridine, respectively.

Reagent added in the control medium	Cultivation time (days)	Growth rates* (%)	NAD content per cell (%)
Control	2	100	100
Nicotinamide (10^{-2}M)	2	82	150
INH (10^{-2}M)	2	50	58
3-acetylpyridine (10^{-2}M)	2	51	43

* Growth rates are expressed as per cent of cell count increased in the experimental media to those increased in the control medium.

increase of NAD is not due to the formation of NAD brought about by nicotinamide as the substrate but rather the decomposition of NAD is prevented by the exchange reaction by NADase as already mentioned. Under these conditions of culture, mitosis or proliferation of L cells is decreased to a greater extent along with lapse of cultivation time both in the nicotinamide-treated group and the control than the differences observable between these two groups. It seems that this decrease of cell proliferation is not due to the degeneration or death of the cells but rather due to changed circumstances (especially mutual interaction among the cells) in which the cells are placed with lapse of the cultivation time. This can be understood from the fact that the culture cells regain their active proliferative power when they are transferred into a new medium with a smaller inoculum size several days after the start of the incubation.

This decrease in the rate of growth along with lapse of cultivation time is in no way connected with NAD content per cell both in the nicotinamide-treated group and the control. It has been observed that in the nicotinamide-treated group the cell proliferation decreases along with lapse of cultivation time while the NAD content, which has once increased, decreases gradually from the second or the third day of culture and returns to normal level. In contrast to this, for the purpose to see how the cell proliferation is affected when the intracellular NAD content is specifically decreased within the limit where degenerative change of the cells does not occur, INH and 3-AP have been selected from the pyridine compounds already mentioned. Since we do not know any specific inhibitor to NAD synthesis, though there remain some problems, in this case NADase is chosen for its transglycosidic action. After the INH inoculation the NAD content is decreased and the cell proliferation is incompletely suppressed It is supposed that this decrease of the NAD content is due to the formation of INH analog of the NAD by INH insensitive NADase as mentioned by ZATMAN *et al.*[28,29] The problem whether this inhibition of cell proliferation is caused by the decrease of NAD, INH analog formed or INH itself has not been solved.

When 3-AP is inoculated into the culture medium, a marked inhibition of cell proliferation and a decrease of the NAD content can be seen just as with the INH inoculation. It is clear that 3-AP inoculation does not bring about an irreversible reaction from the observation that the cells regain their proliferative power and actively multiply when the medium exchanged on the second day of culture with a new control medium. In addition, in spite of such a marked decrease of NAD there can be recognized no appreciable change in oxygen uptake when the oxygen consumption of those cells cultured in the medium containing 3-AP is measured in Warburg manometer. As the matter of fact contrary to expectation, such a decrease of NAD does not only promote even temporarily the cell growth but rather it results in a marked fall in the cell growth. As can be seen in MORTON's hypothesis, supposing that mitosis is induced when the decrease of NAD reaches a certain critical low level, it is possible that the NAD level at this critical point is lower than the NAD level of the cells cultivated in the medium containing INH or 3-AP.

From these results, what comes to mind with respect to the relationship between mitosis and NAD is that it is difficult to discuss about this relationship simply from the increase of NAD and the lessening of mitosis due to nicotinamide inoculation, the cell proliferation does not seem to be monistically regulated only with NAD.

SUMMARY

1. Addition of nicotinamide (10^{-2}M) into the culture medium brings about an increase of the NAD content and the inhibition of the growth of L cells in culture. This rise of NAD brought about by nicotinamide lasts for 2 to 3 days, and thereafter gradually subsiding, it returns to normal level.

2. When L cells are cultured for several days in the same medium without addition of nicotinamide, there occurs a slow-down of mitosis with lapse of cultivation time but it has been found that this is in no way connected with the intracellular content of NAD.

3. By the addition of isonicotinic acid hydrazide (10^{-2}M) into the culture medium, there can be recognized a decrease of NAD content in L cell and the inhibition of cell growth.

4. In the case when 3-acetylpyridine (10^{-2}M) is added, a decrease of intracellular content of NAD in L cells and a marked inhibition of the cell growth can be observed. In the groups cultured in the media, containing 3-AP at the concentration of 10^{-3}M or 10^{-4}M can be seen neither inhibition nor acceleration of the cell growth. The oxygen uptake of the cells cultured in the medium containing 3-AP (10^{-2}M) hardly differs from that of the control group cultured in the medium not containing 3-AP.

5. On the basis of these results discussion has been made on the relation ship between mitosis and NAD content in the cell.

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