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Uptake of nicotinamide adenine dinucleotide and excretion of its degradation pro-ducts by tissue culture cells

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Uptake of nicotinamide adenine dinucleotide and excretion of its degradation pro-ducts by tissue culture cells*

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Abstract

For the purpose to reveal the mechanism of uptake and degradation of NAD by cells, the authors conducted the observation on the L cells cultured in the medium containing NAD and the following results have been obtained. 1. NAD in the medium is taken up by the cells in its intact form, reaching about twice the value of the control. 2. The spontaneously degraded products of NAD, nicotinamide and adenine dinucleotide ribose, in the same molar concentration as NAD used in the present experiment, have no effect on the NAD content of L cells. 3. The NAD taken up by the cells is degraded into nicotinamide mononucleotide (NMN) and adenine mononucleotide (AMP) by pyrophosphatase including NADpptase and excreted in the medium. Unexpectedly the ingested NAD is not degraded by NADase in the L cell. 4. L cells metabolize the same amount of NAD as that contained originally in the cell for about ten minutes, as calculated from the amount of NMN excreted in the medium.

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UPTAKE OF NICOTINAMIDE ADENINE DINUCLEOTIDE AND EXCRETION OF ITS DEGRADATION PRODUCTS BY TISSUE CULTURE CELLS

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As is well known, nicotinamide adenine dinucleotide (NAD) plays an important role as a coenzyme in various biological oxidation reactions. Apart from this function attention has been drawn recently to NAD on two other grounds. The one is that NAD might be involved in biochemical control mechanism of mitosis. MORTON[1] (1958) proposed in his report entitled "Enzymic synthesis of coenzyme I in relation to chemical control of cell growth" that the cell division might be regulated with the level of NAD in the cytoplasm. This hypothesis was supported by FUJII and his coworkers,[2,3] who found the intimate relationship between cell division and NAD contents of the cell in various conditions. One of us[4] also observed about the effects of nicotinamide, isonicotinic acid hydrazide, and 3-acetylpyridine on the growth and the NAD content of L cells, whose data were not always consistent with those of FUJII or MORTON.

Another point, to which attention has been called, is its pharmacological action, particularly therapeutic action of NAD. O'HOLLAREN[5,6] (1961) successfully utilized pyridine nucleotides, particularly NAD, in the prevention, alleviation and removal of acute and chronic symptoms in alcoholism and drug addiction of heroin, morphine, codeine, barbiturates, and the like. THÖLEN *et al.*[7] have obtained marked results in the treatment of hepatic coma by administering NAD in conjunction with coenzyme A and α-lipoic acid. There are also several clinical reports[8,9,10,11] dealing with usefulness of NAD in liver damages as medicament, and with its vasodepressor activity, etc. In dicussing the mechanism of these biological actions of NAD it is very important to study about the uptake and metabolism of NAD by cells. There is, however, little information about the fundamentals of biological activity of NAD.

With the purpose to reveal the mechanism of uptake of NAD by cells and the process of its metabolism, observations have been carried out on the L strain cells cultivated in the medium containing NAD by pursuing the changes of intra- and extracellular NAD concentrations and the metabolized products in the in-

cubated media. In the present paper it is demonstrated that the NAD is taken up directly by the cells and split into nicotinamide mononucleotide (NMN) and adenine mononucleotide (AMP).

MATERIALS AND METHODS

The L cells used were of those from the strain of the Infectious Disease Institute of Tokyo University. They were cultured in medium composed of 8 volumes of a saline D[12] containing 0.08% yeast extract from DIFCO and 0.4% lactalbumin hydrolysate from NBC Chemical Co., one volume of bovine serum inactivated at 56°C for 30 min. and one volume of NAD solution of the various concentration from 4.5 to 0.5 mg per ml. The culture media were sterilized through Toyo Asbestos Sterilizing Film No. 85. The NAD used was of Sankyo Co., Ltd., NBC Chemical Co., or Sigma Chemical Co. These were dissolved in distilled water or saline D, containing yeast extract and lactalbumin hydrolysate, in a concentration to be adequate for each observation. For the culture vessels rectangular culture bottles B or TD 15 flasks (the products of Ikemoto Rika Kogyo Co., Ltd.) were used.

The cells were incubated with NAD from 1 hour to 5 days for the longest. NAD content in the cells, and the concentration of NAD and its decomposed products in the media were observed as follows.

For the measurements of NAD and NADH spectrophotometric method was used with alcohol dehydrogenase, which was extracted from baker's dry yeast (Oriental Yeast Co., Ltd.) by the method of RACKER[13]. After harvesting the cells they were separated from the media by centrifugation at 0°C, 4000 r. p. m. for 10 min. The cells were washed for 3 times at 0°C by repeated centrifugation with saline D containing yeast extract and lactalbumin hydrolysate in the same concentration as that used for the culture medium. The precipitated cells were treated with 5% trichlor-acetic acid (TCA) and ether to extracts NAD by the method described in the previous paper[4]. The measurements were carried out at 340 mμ for 3 times; 1st measurement without adding alcohol dehydrogenase (R_1), 2nd measurement adding the enzyme (R_2), and 3rd measurement after the supplemental addition of the enzyme (R_3). Just before the each measurement the samples were centrifuged in order to diminish the turbidity occasionally observable. From the obtained values the absorbance (R) due to the reduction of the NAD contained in the extract was obtained by the equation: $R \fallingdotseq R_2 - R_1 - (R_3 - R_2)$. The quantity of NAD of the extract was calculated from this R by using millimolar extinction coefficient for NAD (6.2)[14]. For the detailed method of the measurement refer to the previous paper[4]. The measurements of NAD content of culture media were carried on the supernatants according to the same principle as in the precipitated cells.

For the substances produced by the degradation of NAD the absorption at 260 mμ was measured on the acid soluble fraction by the following procedure. An equal volume of 1 N perchloric acid (PCA) was added to the cell free supernatant and centrifuged at 0°C, 4000 g for 20 min. The supernatant is diluted by the addition of 14 volumes of distilled water and the absorbance at 260 mμ was measured. Though the absorbance at 260 mμ was found to be small in the control medium in the present assay condition, the absorbance of the control group was deducted from that of the experimental group in order to eliminate absorbance of the control group that is not associated with added NAD. To compare this value with the concentration of NAD and cyanide addition compounds, the absorbance at 260 mμ was calculated as NAD by using millimolar extinction coefficient 17 at 260 mμ[15].

For the detection of the nicotinamide compounds having quarternary nitrogen, which was found in the acid soluble fraction, KCN was used, by which the compounds turn to the cyanide addition compounds having the specific absorbance at 325 mμ. To 0.5 ml of the extract obtained by the same method as in the extraction of NAD 2.5 ml 1 N KCN solution was added and the absorbance at 325 mμ was measured. Just the same meaning as the absorbance at 260 mμ the absorbance at 325 mμ of the control was deducted from that of experimental group. The value thus obtained was calculated as NAD by using millimolar extinction coefficient 6.3 at 325 mμ[16].

For the further detailed analysis of the decomposed products of NAD the column chromatography was carried out by using Dowex 1-formate column. Dowex 1-formate resin was prepared from the Dowex 1-x2 chloride resin (200—400 meshes) by treating with 1 M ammonium formate and 3 N formate (v/v). This was used as 37×0.72 cm^2 column. Six ml of the medium was added with 1 N PCA to make 0.1 N in its final concentration. After keeping at 0°C for several minutes it was centrifuged and deproteinized. The pH of the supernatant was adjusted to 8.0—8.5 with 5 N KOH, and recentrifuged. Six-seven ml of the supernatant were absorbed to the Dowex column and eluted with increasing concentration of formic acid and ammonium formate solutions by employing the method of TERADA[17]. The remaining supernatant was used to obtain the total absorbance at 260 mμ, the quantities of cyanide addition compounds, and NAD contents. Eluant mixing vessel was of 100 ml volume and the volume of eluate in each tube was about 5.6 ml, though the volume differed within the range 5.5—5.8 ml with each experiment. The elution is conducted with 20 ml of distilled water, 40 ml of 1 N formic acid, 180 ml of 4 N formic acid, 200 ml of 4 N formic acid-0.2 M ammonium formate (v/v), 80 ml of 4 N formic acid-0.4 M ammonium formate (v/v), 200 ml of 4 N formic acid-0.8 M ammonium formate (v/v), and 80 ml of 4 N formic acid-2.0 M ammonium formate (v/v), in the

order mentioned.

Identification of the principal peaks of eluates at E_{260} was made by comparing the concentrations of eluants at a given site of elution and E_{280}/E_{260} ratio with those of the original method[17] as well as with the table of Pabst laboratory[14]. Of the fractions that showed the high absorbance at 260 mμ the quantitative estimations of cyanide addition compounds, ribose by orcinol reaction, and organic phosphate by Allen's method were made.

RESULTS

Observations on the cell growth and changes in the intracellular NAD content of the L cells cultured in the medium containing NAD at high concentrations (400 γ/ml) for 1 to 4 days revealed the depressed cell growth and a marked increase in the NAD level in the cells, but not so clear correlation between the growth suppressing rate and the intracellular NAD concentration (Table 1). The NAD contents in the experimental group rose markedly, reaching about double those of the control group. As the NAD level in the cells cultured in the control medium showed a considerable variety all the assays were made with the accompanied controls. In the low concentrations of NAD (157 γ and 81 γ/ml) of the medium, only a slight increase in the intracellular NAD content was recognized as compared with the control. The suppression of cell growth was hardly seen. The NAD concentration in the media gradually diminished with lapse of time, as illustrated in the table. Almost the same results were obtained with *Sankyo's* NAD and that of NBC Chemical Company.

Hourly observations on the NAD content in the cells showed that by 4 hours after adding NAD in the media the NAD level in the cells reached the maximum value, about 200% of the control level (Table 2a and 2b). The values obtained at one hour incubation with NAD, showed about one third of the maximum value suggesting that it reached the maximum after about 3 hours (Table 2a, 2b). These experiments were conducted on the cells grown for two days on the control medium containing no NAD.

The cells having NAD in a high concentration by incubating with the medium containing NAD for 23 to 24 hours lost their NAD rapidly when they were placed in the same medium with exception of NAD, namely after about 10 hours they lost about 70% of the increased NAD. After 21 hours incubation with the medium having no NAD the NAD level was lowered to the normal level found in those of control group (Table 3). The controls were cultured with the same media without NAD.

As just demonstrated the NAD concentration in the extracellular fluid decreased with the time of incubation. The decrease in NAD might be estimated at each incubation period as the amount of the cyanide addition compound. But

Table 1 Effects of NAD on the growth and NAD content of L cells
Experimental culture medium contained NAD. Control culture medium was of the same composition without NAD.

Culture condition	Amount of NAD administered (μg/ml)	Cultivation time (hrs)	Final NAD amount in cul. med. (μg/ml)	NAD content		Growth rates** (%)
				per cell (μμg)	per g. f. c.* (μg)	
Exp.	406	46	160	1.71	312	76
Cont.	0	46	0	0.87	156	100
Exp.	406	69	139	1.56	347	68
Cont.	0	69	0	0.57	121	100
Exp.	406	92	111	1.09	266	73
Cont.	0	92	0	0.56	143	100
Exp.	380	24	280	1.36	284	106
Cont.	0	24	0	0.87	184	100
Exp.	380	44	211	1.37	244	79
Cont.	0	44	0	0.85	159	100
Exp.	380	75	130	1.18	316	102
Cont.	0	75	0	0.61	168	100
Exp.	327***	25	203	1.52	324	64
Cont.	0	25	0	0.61	145	100
Exp.	327***	70	118	1.26	365	56
Cont.	0	70	0	0.59	180	100
Exp.	157	25	77	0.87	265	92
Cont.	0	25	0	0.66	180	100
Exp.	157	69	26	0.70	178	107
Cont.	0	69	0	0.55	132	100
Exp.	157	97	13	0.85	202	87
Cont.	0	97	0	0.65	166	100
Exp.	81	24	20	0.69	202	113
Cont.	0	24	0	0.52	148	100
Exp.	81	48	6	0.87	225	90
Cont.	0	48	0	0.52	135	100

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

** Growth rates were expressed in per cent of cell counts increased in the experimental medium to those increased in the control medium.

*** In these experiments NAD was the product of NBC Chemical Company. In the other experiments NAD was the product of SANKYO Co., Ltd.

Table 2 Hourly changes of the NAD content in the L cells incubated in the medium containing NAD
In the experiment (a) NAD was the product of SANKYO Co., Ltd., in the experiment (b) NAD was the product of Sigma Chemical Company.

Culture condition		Amount of NAD administered (μg per ml)	Time treated with NAD (hrs)	NAD content	
				per cell (μμg)	per g. f. c. (μg)
(a)	Exp.	383	1	1.01	214
	Cont.	0	1	0.76	160
	Exp.	383	5	1.49	290
	Cont.	0	5	0.74	152
(b)	Exp.	460	1	1.26	208
	Cont.	0	1	0.89	150
	Exp.	460	4	1.69	342
	Cont.	0	4	0.79	150
	Exp.	460	7.5	1.86	381
	Cont.	0	7.5	0.87	191

Table 3 Changes in NAD content of L cells cultivated at first in the medium containing NAD, washed with Hanks' solution and then incubated with the medium containing no NAD.
NAD from NBC Chemical Company was used.

Culture condition		Amount of NAD administered (μg/ml)	Cultivation time (hrs)	Final NAD amount in cul. med. (μg/ml)	NAD content	
					per cell (μμg)	per g. f. c. (μg)
(a)	Exp.	337	23	230	1.63	379
	Cont.	0	23	0	0.80	166
			23*			
	Exp.		33	0	1.12	225
	Cont.		33	0	0.90	201
(b)	Exp.	337	24	223	1.15	310
	Cont.	0	24	0	0.68	188
			24*			
	Exp.		45	0	0.82	213
	Cont.		45	0	0.76	206
	Exp.		97	0	0.62	199
	Cont.		97	0	0.59	208

* Both experimental and control culture media were changed at this time with the control medium.

Table 4 Daily changes of NAD, cyanide addition compounds and absorbance at 260 mμ in the medium cultivating L cells.

	Time after NAD administration (hrs)	Amount of NAD in cul. med. (μg/ml)	Cal. value of CN add. comp. *as NAD (μg/ml)	Cal. value of absorb.** at 260 mμ as NAD (μg/ml)
(a)	0	399	430	551
	22	337	403	541
	46	274	395	538
	76	193	391	526
	101	160	403	534
	117	124	393	538
(b)	0	372	408	498
	24	258	358	476
	48	190	324	472
	72	154	299	456
	96	112	289	433
	120	100	290	440

* CN add. comp.: abbreviation of cyanide addition compounds.
** Cal. value of absorb.: abbreviation of calculated value of absorbance. Same abbreviations are used in the following tables.

Table 5 Daily changes of NAD, cyanide addition compounds and absorbance at 260 mμ of NAD solution, dissolved in the culture medium containing no cells (a) or in distilled water (b), and incubated at 37°C. Exp. 5a was done with the same condition as Exp. 4b with exception of L cell inoculation.

	Time after incubation	Amount of NAD in solution (μg/ml)	Cal. value of CN add. comp. as NAD (μg/ml)	Cal. value of absorb. at 260 mμ as NAD (μg/ml)
(a)	0 (hrs)	372	404	500
	24 〃	268	303	480
	48 〃	146	187	464
	72 〃	94	156	455
	96 〃	74	150	444
	120 〃	58	138	440
	144 〃	47	129	400
(b)	0 (days)	4080	4310	6120
	3 〃	3730	3890	4880
	7 〃	3460	3520	4910
	11 〃	3080	3240	5150
	20 〃	2340	2580	5190
	28 〃	1990	2160	5320
	42 〃	1320	1480	4970
	59 〃	890	1070	5220

the analysis of the acid soluble fractions proved that the amount of the substances detected as the cyanide addition compounds did not so markedly decrease as that of NAD, while in the medium incubated free of cells the decrease in the cyanide addition compounds showed a good coincidence with the decrease in NAD (Table 4b and 5a). Experiment illustrated in Table 5a was conducted under the same condition as that illustrated in Table 4b with exception of L cell inoculation. Total amount of the bases detected by the absorbance at 260 mμ showed only a slight decrease throughout the cultivation for 5 days (Table 4a, 4b, 5a). Further analyses of the acid soluble fraction of the culture media incubated with cells by the column chromatography revealed the formation of adenine mononucleotide (AMP) and nicotinamide mononucleotide (NMN) with the decrease of NAD (Fig. 1a, 1b). While in the similar fraction of the culture

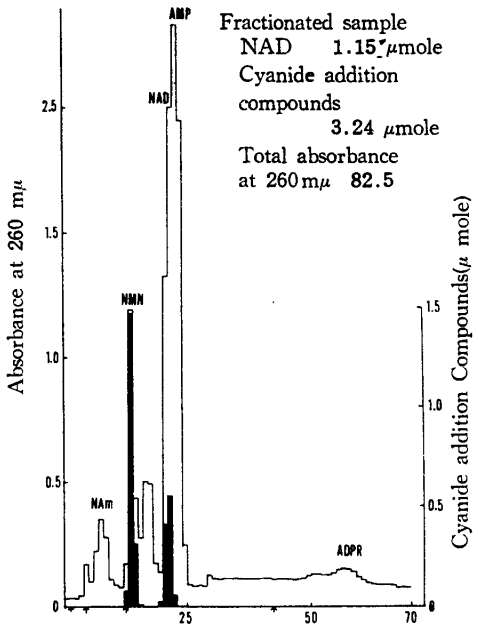

Fig. 1 a Elution pattern of acid soluble fractions from the mixture of media taken on the second, third, fourth, fifth day of the culture illustrated in Table 4a. White column shows the absorbance at 260 mμ. Black column shows the molar content of cyanide addition compounds. Same expression is also used in the following figures.

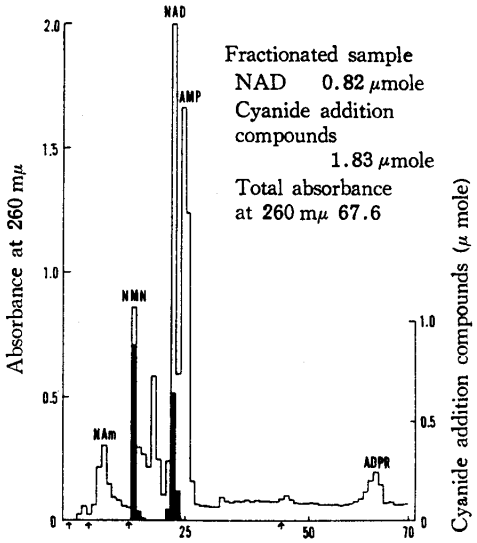

Fig. 1 b Elution pattern of acid soluble fractions of the medium on the third day of culture shown in Table 4b.

media incubated without cells showed the increase in adenine dinucleotide ribose (ADPR) and nicotinamide with the decrease of NAD (Fig. 2). NMN and AMP could be detected only slightly. Among those substances formed by the decomposition of NAD the NMN only can be detected as the cyanide addition compound. Namely, the total amount of cyanide addition compounds of the fractionated samples was recovered in the fraction of NAD and NMN. Observations on the natural decomposition of NAD in the distilled water as observed by incubating for a long period till 59 days at 37°C showed about the similar feature as

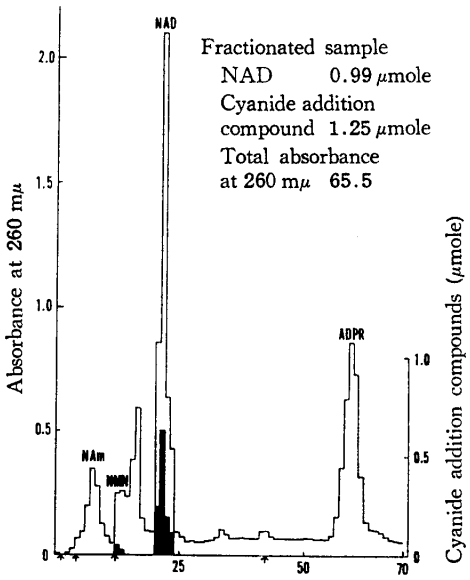

Fig. 2 Elution pattern of acid soluble fractions of the medium on the two days after NAD addition shown in Table 5a.

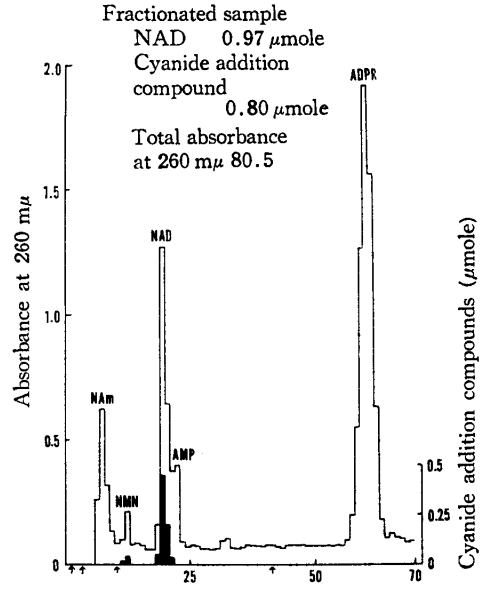

Fig. 3 Elution pattern of the sample shown in Table 5b on the 59th day of incubation.

in the cases incubated for a short period in the culture media without L cell inoculation demonstrating the decrease of NAD with the compariable decrease in cyanide addition compounds (Table 5b and Fig. 3).

The possibility of the new synthesis of NAD from the degraded products of NAD was tested by observing the changes in the amount of NAD in the cells and cyanide (CN) addition compounds in the medium incubating the cells for 3 to 5 days with the partially degraded products of NAD. In the case incubated with the degraded product obtained at 59th day of incubation (Fig. 3) it was shown that the amount of CN addition compounds in the medium did not

increase any more than what can be explained as a part of residual intact NAD that has been ingested and decomposed by the cells, suggesting no new synthesis of NAD in the cells from the degraded materials (Table 6). Column chromatographic analysis of the medium at 3rd day of incubation showed that ADPR disappeared with the increase in AMP showing ADPR was taken up by the cells and excreted mainly as AMP (Fig. 4). Analyses of the cells and the culture medium proved again no more increase in NAD content of the cells and CN addition compounds in the medium than what could be explained due to residual intact NAD (Table 7).

Table 6 Daily changes of NAD, cyanide addition compounds and absorbance at 260 mμ in the culture medium of L cells, in which partially decomposed NAD was administered initially.

Time after administration (days)	0	2	3	4	5
Amount of NAD (μg per ml)	89	39.6	21.8	13.4	7.04
Cal. value of CN add. comp. as NAD (μg per ml)	107	89.6	86.0	87.2	89.6
Cal. value of absorb. at 260 mμ as NAD	522	473	447	436	408

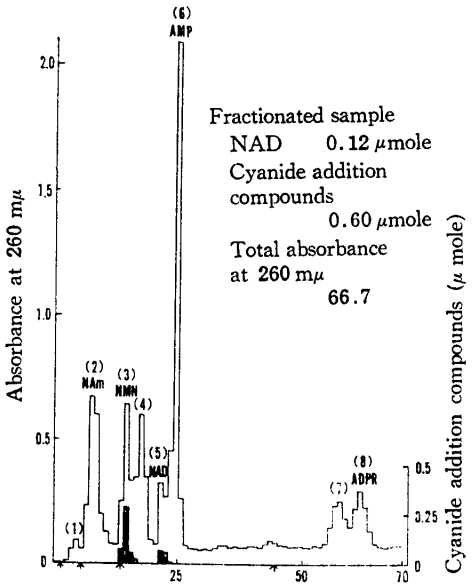

Fig. 4 Elution pattern of the acid soluble fractions of the medium on the third day of culture shown in Table 6.

The purity of NAD sample tested by the analysis of NAD, CN addition compounds and absordance at 260 mμ, and by the column chromatography proved the good quality adequate for the present study (Fig. 5).

The identification of each peak in the column chromatographies was made by the methods already mentioned, and the indirect methods for the quantitative analysis of nicotinamide compounds and adenine compounds. The data for NMN, AMP, and ADPR are shown in Table 8. Fraction 2 is thought to be nicotinamide by the fact that the site where it is eluted (the pH of eluate, 4.7) and the absorbance at 260 mμ at this pH are fairly proportional to the nicotinamide formed by the decomposi-

Table 7 Effects of partially degraded NAD on the content of NAD of L cells and daily changes of NAD, cyanide addition compounds and absorbance at 260 mμ in the culture medium.

Experimental condition		Degraded NAD	NAD	Control	Degraded NAD	NAD	Control
Cultivation time (hrs)		26	26	26	69	69	69
Initial amount in the medium	Amount of NAD administered (μg/ml)	65	372	0	65	372	0
	Cal. value of CN add. comp. as NAD (μg/ml)	99.8	404	0	99.8	404	0
	Cal. value of absorb. at 260 mμ as NAD (μg/ml)	533	500	0	533	500	0
Final amount in the medium	NAD (μg/ml)	33.2	194	0	23.0	103	0
	Cal. value of CN add. comp. as NAD (μg/ml)	84.6	332	0	86.5	311	0
	Cal. value of absorb. at 260 mμ as NAD (μg/ml)	516	468	0	483	432	0
NAD content per cell (μμg)		0.98	1.98	0.86	0.73	1.94	0.84
NAD content per gram of fresh cells (μg)		177	284	132	125	271	128

tion of NAD. Fraction 4 is the compound contained in the yeast extract. Fraction 7 is thought to be cyclic AMP excreted after uptake and metabolism of the ADPR by the cells, though these facts were not determined.

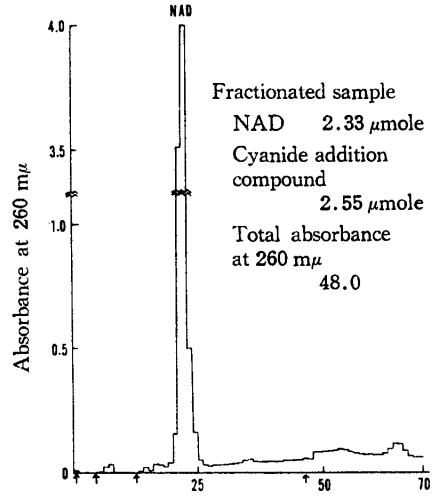

Fig. 5 Elution pattern of NAD (SANKYO) used in this experiment.

DISCUSSION

As described previously, there are many reports dealing with pharmacological action of NAD. However, as has been pointed out by MIYAGI[18] not only its action mechanism but also the permeability of NAD to cell membrane have not been studied precisely, though KAPLAN and associate[19], and MINARD and others[20] reported the rise of NAD contents in various organs of mice after NAD administration, suggesting the direct uptake of NAD by the liver cells.

Table 8 Identification of the compounds, eluted from Dowex-1-formate column, with molar ratios of components.

Fig. No.	Tube No.	Nicotinamide content calcu. from CN add. compound	Adenine content cal. from absorb. at 260 mμ	Ribose (Olcinol method)	Phosphate (Allen's method)	Identified compounds
1 a	14 (13—15)	1	0	0.93	1.00	NMN
1 b	15 (15—16)	1	0	0.94	1.03	NMN
1 a	24 (23—25)	0	1	1.04	1.05	AMP
1 b	26 (25—27)	0	1	1.03	1.08	AMP
4	25 (23—25)	0	1	0.96	1.09	AMP
2	60 (56—64)	0	1	2.16	2.16	ADPR
3	61 (57—64)	0	1	1.86	2.05	ADPR

The time required for the uptake to its maximum level was assumed as 2 hours in mouse liver by KAPLAN *et al*[19]., 6 hours, MINARD *et al*[20]., and 3—4 hours in the cultured mouse fibroblasts (L cells) by our observation. This is also the case in the administration of NAD-precursors such as nicotinamide, nicotinamide mononucleotide, and nicotinic acid as demonstrated by MINARD and others.[20] However, NAD decomposes at a considerable speed when it is dissolved in water or medium without cell and incubated at 37°C. This fact shows the possibility that NAD decomposes first and the decomposed substances are taken up by cells only for the resynthesis of NAD. But in the present study on L cells suggests the direct uptake of NAD in its intact form. The NAD level in L cells solely depends upon the NAD level in the medium, but not the concentration of the decomposed substances. The cellular contents of NAD are found always to be proportional to the amount of NAD in the extracellular fluid at the time when the cells are harvested. The cellular NAD level does never rise when the cells have been incubated with the decomposed substances of NAD, which are found in the medium containing NAD after a long term incubation without cells. When the cells are incubated with the partially decomposed products of NAD the nicotinamide level in the medium does not decrease but rather increase, probably due to the spontaneous decomposition of the residual intact NAD contained in it. In contrast, most of ADPR in the medium is obliterated with the increase in AMP, signifying that ADPR is ingested by the cells, split at the pyrophosphate linkage and AMP is excreted outside the cell.

In the cells incubated with the medium of high concentration of NAD (460 γ/ml), the intracellular concentration of NAD was found to be 300 γ/g, which was lower than that in the medium and corresponded about twice of normal value of intracellular NAD concentration (about 160 γ/g), while in the cells incubated with the medium of low concentration of NAD (80 γ/ml), the intra-

cellular level of NAD became 200 γ/g, which was higher than that in the medium. Since intracellular NAD exists either as bound form or as free form, it will not be possible to know the density gradient between intra- and extracellular free forms of NAD in these experiments. For the purpose to learn the mechanism of transport of NAD through cell membrane, effects of 2,4-dinitrophenol (DNP) and ouabain on the uptake of NAD by the cells were tested. As the results neither DNP nor ouabain has significant inhibitory effects on the uptake of NAD by the cells. These results seem to suggest that the uptake of NAD by the cells is not due to active transport but passive transport.

NAD taken up by cells is metabolized by the specific way being decomposed into AMP and NMN and excreted into the medium, while in the medium free of cells NAD is decomposed into nicotinamide (NAm) and ADPR by the hydrolysis at nicotinamide riboside linkage. As for the enzymes that decompose NAD in the cell, nicotinamide adenine dinucleotidase (NADase)[21] and nicotinamide adenine dinucleotide pyrophosphatase (NADpptase)[22] are known. The NADase split NAD into NAm and ADPR just as in the spontaneous decomposition of NAD and is considered to be the most important enzyme among those concerning the catabolism of NAD in the living tissues[1,23]. SEKI has found this enzyme activity in L cells by the biochemical method[4]. FUJII and his coworkers[2] are of the opinion that the NAD level in the cell is regulated by the cellular NADase activity. However, the present experiment proved that the NAD ingested by L cells is cleaved at pyrophosphate linkage forming NMN and AMP, which are found in the medium in which the cells have been incubated. Though these substances are found only slightly in the medium having no cells, they are markedly found even in the medium having the cells and the NAm level in the latter medium is less than that found in the cell free medium incubated for the corresponding period. The NMN level in molar concentration in the medium having the cells is always less than that of NAD decomposed during the incubation. The results shows that most parts of NAD taken by the cells is decomposed by NADpptase or other phrophosphatase but not by NADase. The lesser degree in decrease of NAD in the medium having L cells comparing to that in cell free medium is probably due to decelerated decomposition of NAD in the former by the marked fall of pH of the medium.

The fact that three hours are required for the intracellular concentration of NAD to reach its maximum appears to show that the turnover of the uptake-decomposition of NAD would be extremely slow. But taking into consideration the increasing rate of NMN in the medium the turnover of NAD will considerably be rapid. The increased amount of NMN in the medium 3 days after NAD addition to the medium containg 0.017 g of wet weight of cells was about 1.14 mg/10 ml, i. e. 1.7 μ moles (Table 4b). It means that 100 μ moles of NAD

have been metabolized by gram of wet cells. As the control cells contain about 0.24 μ moles of NAD per gram of wet cells, one may expect in these cells that about 420 times NAD of the normal level is metabolized during 72 hours, 5.8 times per hours. It means that it requires only 10 minutes to reach the maximum intracellular NAD level, provided that the cells stopped the degradation of the ingested NAD.

SUMMARY

For the purpose to reveal the mechanism of uptake and degradation of NAD by cells, the authors conducted the observation on the L cells cultured in the medium containing NAD and the following results have been obtained.

1. NAD in the medium is taken up by the cells in its intact form, reaching about twice the value of the control.
2. The spontaneously degraded products of NAD, nicotinamide and adenine dinucleotide ribose, in the same molar concentration as NAD used in the present experiment, have no effect on the NAD content of L cells.
3. The NAD taken up by the cells is degraded into nicotinamide mononucleotide (NMN) and adenine mononucleotide (AMP) by pyrophosphatase including NADpptase and excreted in the medium. Unexpectedly the ingested NAD is not degraded by NADase in the L cell.
4. L cells metabolize the same amount of NAD as that contained originally in the cell for about ten minutes, as calculated from the amount of NMN excreted in the medium.

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