Table 1. *Polarographic estimation of DPA in the presence of (a) spores (method 1) and (b) vegetative cells in broth (method 2)*

Supporting electrolyte for method 1 was citric acid-phosphate buffer, pH 3.3, and for method 2 was 0.5N-HCl.

	Concn. of DPA ($\mu\text{g./ml.}$)		Difference (%)
	Weighed	Estimated	
(a)	2.5	2.55	2.0
	6.0	6.15	2.5
	12.0	12.13	1.2
	17.0	17.20	1.1
	29.0	29.30	1.0
(b)	2.5	2.60	4.0
	5.5	5.70	3.6
	10.5	10.30	1.9
	12.0	11.90	0.8
	27.0	27.30	1.1

determinations with fresh solutions made from crystalline DPA for each determination.

Effect of spores, nutrient broth and vegetative cells on DPA determination. *Bacillus stearothermophilus* N.C.I.B. 8919 spore suspensions in water (Cook & Brown, 1964) to a final concentration of about $5 \times 10^7/\text{ml.}$ were added to known concentrations (weighed) of DPA and assays were made by method 1.

An equal volume of a broth culture of vegetative cells was added to known concentrations (weighed) of DPA with HCl to 0.5N and assayed by method 2.

Table 1 shows that the assay remained sensitive

in the presence of spores, broth and vegetative cells. In no case was the difference between the assay estimate and the weighed amount greater than 4%. A direct comparison of the two methods was made by autoclaving (5 hr., 25 lb./in.²) a spore suspension of total (chamber) count $5 \times 10^8/\text{ml.}$ to release DPA (Janssen *et al.* 1958) and assaying by methods 1 and 2. This procedure was repeated to test complete release of DPA with the same suspension. There was no significant difference between the methods for DPA release by autoclaving. The mean value ($\mu\text{g./ml.}$ of spore suspension) was 68.4 ± 0.8 (8). Twin cell subtractive operation confirmed that spores did not interfere with the DPA determination with either method.

Spores release DPA in the presence of HCl (Janssen *et al.* 1958) and consequently method 2 was found to be less useful than method 1 in the presence of spores still retaining some DPA. Delay in polarographing the solutions under these circumstances led to gradually increasing readings, owing to DPA release. Both methods were satisfactory when used in the presence of spores from which the DPA was totally released. Each calibration graph was prepared in about 40 min. and a single determination took about 6 min.

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The Pathway of Synthesis of Mammary-Gland Nicotinamide-Adenine Dinucleotide

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Although it is now generally thought that the main pathway of NAD synthesis is that described by Preiss & Handler (1958), the existence of this pathway has been demonstrated in relatively few tissues [liver and erythrocytes (Preiss & Handler, 1958); *Escherichia coli* (Ismande, 1961)]. More recently,

interest has revived in the alternative route by which nicotinamide is converted into NMN and hence into NAD, and evidence has now been produced (Grunicke *et al.* 1966) that this latter route is indeed the main mechanism for NAD synthesis in ascites-tumour cells.

Fig. 1. Increase in liver and mammary-gland NAD content after administration of either nicotinamide (a) or nicotinic acid (b). Lactating rats were injected with either nicotinamide (200mg./kg.) or nicotinic acid (30mg./kg.), and killed at the times shown. O, Mammary gland; ●, liver. Control values (zero time) were: liver, 547 μ g. of NAD/g. wet wt. of tissue; mammary gland, 214 μ g. of NAD/g. wet wt. of tissue.

In investigating NAD synthesis in mammary tissue the enzymic findings appeared to indicate that the Preiss & Handler (1958) pathway either did not operate in this tissue or had an activity far too low to account for the observed rates of synthesis, and it therefore became important to investigate alternative routes. Advantage was taken of the observation by Ijichi, Ichiyama & Hayaishi (1966) that, in rats, injected nicotinamide was rapidly excreted into the gut and then subsequently re-absorbed as nicotinic acid, which returned to the liver and was incorporated into NAD via the Preiss & Handler (1958) pathway.

Lactating rats (body wt. 250–270g.) were taken on the fourteenth day of lactation and injected with either nicotinamide (200mg./kg.) or nicotinic acid (30mg./kg.) and killed at fixed intervals thereafter. Those rats that were to be killed at the shorter times after injection (up to 20min.) were injected intravenously, and those killed after an interval of greater than 20min. were injected intraperitoneally. At the intervals shown in Fig. 1, the rats were killed by cervical dislocation and 200–300mg. samples of mammary gland and liver removed. These samples

were extracted in either hot 0.1N-HCl or 0.1N-NaOH as described by Glock & McLean (1955). The extracts were neutralized and centrifuged and the NAD⁺ and NADH estimated in the extracts, by using an oxygen electrode as described by Greenbaum, Clark & McLean (1965). The results of this experiment are shown in Fig. 1.

In the nicotinamide-treated rats it is apparent that, though the mammary-gland NAD content rises rapidly and reaches a plateau value by 30min., the hepatic NAD content shows no appreciable change during this time and only rises after a lag of 30min. or more, the rise then continuing for several hours (not shown). The delayed rise in hepatic NAD content is consistent with the results of Ijichi *et al.* (1966), who showed that the nicotinamide had first to be deamidated in the gut before it could be incorporated into liver NAD, and that the process of excretion into the gut, deamidation and resorption took some 30min. The very rapid rise in mammary-gland NAD content after nicotinamide treatment could indicate either that deamidation is not a necessary preliminary to NAD synthesis in this tissue or, alternatively, that mammary gland possesses an active deamidase. Two observations indicate that the former explanation is the more likely. The first is that the mammary-gland NAD content achieves a plateau value after 30min., i.e. at a time when the blood nicotinamide concentration is falling and there is a rising blood nicotinic acid concentration as shown by the results of Ijichi *et al.* (1966), and the fact that the liver NAD content is increasing at a time when the mammary-gland NAD content is unchanging. Secondly, when nicotinic acid is injected in place of the nicotinamide it is clear that, although the liver can utilize this substrate readily and with virtually no time lag, the mammary gland cannot and there is no rise in the mammary-gland NAD content at any time after nicotinic acid injection.

The results of this experiment appear to indicate that the mammary gland synthesizes NAD from nicotinamide and that it does so without deamidating it to nicotinic acid. Isotopic and enzymic evidence supporting this conclusion has also been obtained (S. Pinder & A. L. Greenbaum, unpublished work).

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