Comparative Rates of Synthesis of Diphosphopyridine Nucleotide by Normal and Tumour Tissue from Mouse Mammary Gland: Studies with Isolated Nuclei

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(*Received* 23 *November* 1955)

At least three contributory factors determine the development of carcinoma of the mammary gland of the mouse: (1) a genetic susceptibility; (2) a hormonal stimulation normally induced by fertilization; (3) a 'milk factor' transmitted to the offspring via the mother (see Dmochowski, 1953; Greenstein, 1954). It seems possible that this agent is carried into the milk in association with lipoprotein particles called 'milk microsomes' (Morton, 1953, 1954). However, little is yet known of the nature of the milk factor or of the mechanism by which it so greatly accelerates tumour development in genetically susceptible, non-virgin mice. The work described here is part of a general programme to determine the enzymic changes which may accompany tumour development in the mammary gland, with the principal aim of finding the locus of action of the milk factor.

Kornberg (1950) demonstrated the synthesis of diphosphopyridine nucleotide (DPN) from adenosine triphosphate (ATP) and nicotinamide mononucleotide (NMN) according to the reaction:

$$ATP + NMN \rightleftharpoons DPN + PP_i,$$

where PP_i represents inorganic pyrophosphate. In mouse liver, the enzyme which catalyses this reaction (DPN pyrophosphorylase) is localized in the nucleus (Hogeboom & Schneider, 1952). Upon DPN (and the derived triphosphopyridine nucleotide, TPN) depend many cytoplasmic dehydrogenases (see Racker, 1955, for a tabulation). The aerobic generation of ATP in animal tissues is largely from oxidation of reduced DPN and reduced TPN (Hunter, 1951; Lehninger, 1951). Hence many synthetic reactions (including protein synthesis), which directly or indirectly utilize ATP, are ultimately DPN-dependent. The rate of supply of products derived from an enzyme system localized in the nucleus may thus markedly influence cytoplasmic reactions; disturbance of such a system might well have a profound influence on the behaviour of the cell, particularly on cell division and differentiation.

On the basis of this hypothesis, the relative DPN-pyrophosphorylase activities of nuclei isolated from normal mammary gland and tumour of the mouse have been determined. It has been found that the rate of synthesis of DPN by tumour nuclei is only about one-fifth of that of nuclei from lactating mammary glands. Preliminary results (unpublished) indicate that the DPN pyrophosphorylase is localized in the nucleus in both normal mammary gland and tumour, so that the comparative results reflect the different rates of synthesis by the two types of tissue.

MATERIALS AND METHODS

Animals

The strain of mice with high tumour-incidence (C_3H) was obtained from the Chester Beatty Cancer Research Institute, London. The low tumour-incidence (NZ) strain was obtained from the Medical School, University of Otago, Dunedin, New Zealand. Both colonies were maintained (by continued inbreeding) in the laboratory for about 12 months before this investigation was begun. In the C_3H colony, almost all the non-virgin females in each generation surviving to tumour-bearing age have developed tumour of the mammary gland, while no case of spontaneous mammary gland tumour has yet been observed in the NZ strain. The mice were kept in galvanized or tinned-iron cages. The diet consisted of food pellets ('Fido' dog cubes, Barastoc Products Ltd., Victoria) and water, supplemented with fresh cabbage leaf weekly, and occasionally with raw sheep's liver.

Reagents

Nicotinamide from L. Light and Co., adenosine-5′ phosphate from Roche Products Ltd., and DPN from Sigma Chemical Co. ('Cozymase 90') were used without further purification.

Adenosine triphosphate. ATP was obtained as the sodium salt from Gedeon Richter Ltd., Budapest, Hungary. It assayed as 58% ATP by the enzymic method of Slater (1953). The preparation was found to be heavily contaminated with DPN and metals, and inhibited DPN synthesis. The inhibition was removed during the following purification treatment. About 3 g. of ATP was dissolved in 20 ml. of water, brought to pH 6·5–6·8, and H_2S passed through the solution until no further precipitate appeared. The precipitate was removed by filtration, first through no. 1 Whatman paper and then through a thin layer of Hyflo Supercel overlying a no. 42 Whatman paper on a Büchner filter funnel. Excess of barium acetate was added, the barium salt of ATP washed four times with 0·1 M sodium

acetate buffer, pH 6·0, and dissolved in 4 N-HCl and converted into the sodium salt by adding sodium sulphate and adjusting to pH 6·8. The barium sulphate was removed by centrifuging, and the supernatant (approx. 10 ml.) used for the enzymic work.

In a single case, the rate of synthesis of DPN with this purified ATP was compared with that obtained with crystalline sodium ATP (Sigma Chemical Co.). No significant difference in rates was observed.

Alcohol dehydrogenase. Initially, the crystalline enzyme was prepared from dried Fleischmann's yeast (kindly supplied by Dr E. Racker) by the method of Racker (1950). Later, it was obtained from Boehringer and Soehne, Mannheim, Germany.

Potato pyrophosphatase. A survey of several locally grown varieties of potatoes (both 'old' and 'new'-season crops) showed that the maximum activity was no more than one-tenth of that found in the very active American varieties by Kornberg & Pricer (1950). The enzyme was initially prepared by the method of Kornberg & Pricer (1950), but the activity at all stages was considerably less than that obtained by these workers. The following modification of their procedure gave a final preparation of fairly low activity/mg. dry wt., but sufficiently free from contaminating enzymes to give almost quantitative conversion of DPN into nicotinamide mononucleotide.

Approx. 25 kg. of potatoes were peeled, diced, and 100 g. portions dispersed in a Waring Blendor for 90 sec. with 200 ml. of 0·4 saturated $(NH_4)_2SO_4$ solution at about 2°. The portions were combined, and filtered overnight at about 2° through large, fluted no. 1 Whatman papers. Then 6·11 kg. of solid $(NH_4)_2SO_4$ was dissolved in the filtrate (30·6 l.), the suspension held for about 3 hr. and the precipitate collected by centrifuging (approx. 3000 *g* for 20 min.). The precipitate was extracted with about 3·5 l. of 0·25 saturated $(NH_4)_2SO_4$ solution (at about 2°), and the insoluble residue removed by centrifuging as above. About 1·3 kg. of solid $(NH_4)_2SO_4$ was added to 4 l. of the supernatant, the precipitate collected by centrifuging as before, and dissolved in a minimum of glass-distilled water. The solution was dialysed in Visking tubing against running tap water for 2 hr., after which the dialysed material (2·4 l.) was brought to pH 4·5 by the slow addition of 10·5 ml. of N acetic acid. After cooling to −3°, 750 ml. of 95% ethanol (at −10°) was added, and the precipitate collected and dissolved in distilled water (at 0°), the final volume being about 1 l. This solution was adjusted to pH 5·0 and held for 48 hr., at 0°, after which a slight precipitate was removed by centrifuging and the clear supernatant fractionated by successive additions of 95% ethanol as described by Kornberg & Pricer (1950). Most of the activity was fairly uniformly distributed among the first seven fractions, which were combined, dissolved in a minimum of distilled water, and insoluble material was removed by centrifuging (3000 *g*, 30 min.). Alumina gel (58 ml. of C*y*, aged for about 2 years) was added to 724 ml. of solution, and after 30 min. was removed by centrifuging as above. The alumina precipitate was washed four times with successive 25 ml. portions of 0·1 M potassium phosphate buffer, pH 7·4. The enzyme was eluted with two 25 ml. portions of 0·25 saturated $(NH_4)_2SO_4$ solution. Then 9·7 g. of solid $(NH_4)_2SO_4$ was added to the combined eluates (46 ml.), the precipitate collected, dissolved in about 2 ml. of distilled water, and the brown solution clarified at about 25000 *g* for 15 min. The final solution had an activity of 25·8 μmoles of DPN cleaved/hr./ml. at 38°, and an optical density of 11·7 at 280 mμ., corresponding to about 2 μmoles of DPN cleaved/hr./mg. of protein. The DPN appeared to be quantitatively converted into nicotinamide mononucleotide (see below).

Preparation and assay of nicotinamide mononucleotide

Nicotinamide mononucleotide was prepared by the action of potato pyrophosphatase on DPN by the following modifications of the procedure of Kornberg & Pricer (1950). About 45 mg. of DPN was dissolved in 0·4 ml. of 0·2 M potassium phosphate buffer (pH 7·4); 0·1 ml. of 6 M nicotinamide was added, followed by 0·5 ml. of a suitable dilution of potato pyrophosphatase. After adjusting the pH to 7·0, the solution was incubated at 38° for about 2 hr., at which time the DPN had been totally destroyed. Then 0·2 ml. of 72% $HClO_4$ was added, the fine protein precipitate removed by centrifuging (approx. 25 000 *g* for 30 min.), and the supernatant neutralized with 10 N-KOH. The insoluble $KClO_4$ was removed by centrifuging, and washed once with 0·2 ml. of distilled water. The combined supernatant and washings were freeze-dried over conc. H_2SO_4 *in vacuo*. The product was used as nicotinamide mononucleotide without any further purification, since it was shown (see later) that adenosine-5′ phosphate, the other major product of nucleotide pyrophosphatase action, did not interfere with synthesis of DPN.

This product contained no DPN, as determined with alcohol dehydrogenase. The total nicotinamide riboside, estimated by measuring the absorption at 325 mμ. in the presence of approx. M-KCN (Colowick, Kaplan & Ciotti, 1951), corresponded with the amount of DPN destroyed, indicating stoicheiometric cleavage. In some cases, the concentration of nicotinamide mononucleotide was also estimated from the absorption at 340 mμ. after reduction with sodium dithionite (see LePage, 1947).

Determination of the rate of synthesis of DPN

The rate of synthesis of DPN was estimated by the following modification of the procedure of Kornberg (1950). The reaction mixture (0·8 ml., final pH 7·4) contained buffer (0·3 ml. of 0·25 M glycylglycine, pH 7·4), nicotinamide mononucleotide (3 μmoles), ATP (5 μmoles), $MgCl_2$ (15 μmoles) and nicotinamide (about 700 μmoles). The reaction was started by addition of 0·2 ml. of a suitable suspension of nuclei, and the mixture incubated at 38° for 20 min. with gentle mechanical shaking. Then 0·8 ml. of 20% (w/v) trichloroacetic acid (TCA) was added, the precipitate removed by centrifuging, and washed once with 0·2 ml. of 6% (w/v) TCA. The combined supernatant and washing were adjusted to pH 8·0 by cautious addition of 4 N-KOH and made up to 2·5 ml. in a calibrated narrow-bore tube. The control tube was treated similarly throughout, except that the TCA was added immediately before the addition of the nuclei.

The DPN content of 1 ml. of this final extract was estimated spectrophotometrically at 340 mμ. after reduction with ethanol and alcohol dehydrogenase as described by Kornberg (1950). A Beckman instrument, model DU, was used with 1 cm. cuvettes throughout this work.

Nitrogen. This was estimated by the colorimetric procedure previously described (Morton, 1955), except that the digestion was carried out by addition of 0·2 ml. of 50% H_2SO_4 which contained 1 g. of a catalyst/l., prepared by

grinding together 3·46 g. of $CuSO_4,5H_2O$ and 1·54 g. of SeO_2.

Counting of nuclei. A portion of the suspension of nuclei used for the enzymic assay was diluted (usually 1 in 20) into 0·25 M sucrose–0·0009 M-$CaCl_2$ containing a few drops of 0·001 M methylene blue. The stained nuclei were counted microscopically with a clinical haemocytometer (Levy-type counting chamber). All counts were made by the one operator with the one haemocytometer throughout this work. Each count included the nuclei in eighty small squares of the chamber. This was repeated at least five times with a new sample for each count. The total number of nuclei counted in each preparation ranged from 1584 to 2442 for nuclei preparations from tumours; from 468 to 1896 for preparations from mammary glands; and from 265 to 1488 for preparations from liver. Applying the formula developed by Berkson, Magath & Hurn (1940) for estimating the percentage variation in counts of erythrocytes, the variation was between 5 and 9% for these determinations.

RESULTS

Isolation of nuclei

Normal mammary glands were obtained from lactating females taken at varying stages of lactation. They were removed from their litters just before use. In most cases only the hind glands were used and good yields of nuclei were generally obtained. Several attempts to obtain nuclei from the glands of virgin or non-lactating, non-pregnant mice were unsuccessful, because of the negligible amount of gland tissue. The mice used to obtain nuclei from non-lactating glands were mostly in advanced pregnancy. Histologically, the tumour tissue showed spheroidal cells with very little stroma. Central necrosis was common but only solid, white, peripheral, non-haemorrhagic viable tissue was used for assay.

The following method, based on that described by Hogeboom & Schneider (1952), was developed for isolation of the nuclei from both types of tissue. It has been found to give enzymically active nuclei of good appearance under the microscope.

Mice were anaesthetized with diethyl ether and the mammary gland or tumour was excised. The tissue was transferred immediately into ice-cold 0·25 M sucrose–0·0018 M-$CaCl_2$ solution. After mincing as finely as possible with sharp scissors, the tissue was disrupted for 1–2 min. in 5–10 vol. of the same sucrose–$CaCl_2$ solution with a Potter & Elvehjem (1936)–type homogenizer, consisting of a polyethylene pestle in a smooth-walled glass tube as described in the Appendix.

The tissue dispersion was filtered through a layer of 10xx bolting silk and then 5 ml. was layered over 10 ml. of 0·34 M sucrose–0·0009 M-$CaCl_2$ solution in round-bottom centrifuge tubes (15 ml. capacity). The nuclei were sedimented at approx. 600 *g* for 10 min., with an International no. 1 centrifuge, which was accelerated and decelerated slowly in order to avoid mixing of the layers. The time from start to stop was about 15 min. After removing the supernatant, the sedimented pellet was resuspended in 5 ml. of 0·25 M sucrose–0·0009 M-$CaCl_2$ solution by dispersing gently for about 15 sec. with a polyethylene pestle fitting loosely in the centrifuge tube.

The suspension of nuclei was then relayered over 10 ml. of 0·34 M sucrose–0·0009 M-$CaCl_2$ and resedimented as before. The procedure was repeated four times, the pellets from two tubes being combined on each occasion. The final pellet was suspended in about twice its volume of 0·25 M sucrose–0·0009 M-$CaCl_2$.

The whole preparation, including centrifuging, was carried out at 0–1°. The condition of the nuclei was checked at all stages by microscopic examination. As shown in the accompanying plate, the nuclei of the final preparations from both normal and tumour tissue appeared intact.

Assay of DPN synthesis

Preliminary observations established that nuclei isolated from mouse liver, mammary gland and mammary carcinoma synthesized DPN according to the Kornberg (1950) reaction. In routine assays, the reaction was carried out and DPN estimated as described under Methods.

The validity of this assay system was established from earlier investigations with the more active nuclei from mouse liver and lactating mammary gland. With the amount of nuclei normally used (0·1–0·2 ml. of a suspension prepared as previously described) doubling the concentration of ATP or of nicotinamide mononucleotide, or both together, caused no further increase of synthesis in 20 min. at 38°. With the usual incubation period (20 min.), the rate of synthesis was proportional to the amount of suspension of mammary gland or liver nuclei within the normal range used (0·1–0·3 ml. of suspension giving synthesis rates of 0·12–0·36 μmole of DPN/hr./tube). With the amounts of nuclei normally used, the rates of synthesis by liver, mammary gland and tumour preparations were fairly linear up to 1 hr. With prolonged incubation, the rate of synthesis declined, probably due to the reaction approaching equilibrium (see Kornberg, 1950).

Since the nicotinamide mononucleotide used in this work was expected to contain an equivalent concentration of adenosine-5′ phosphate, the effect of excess of the latter on DPN synthesis was studied. It was observed that increase of the concentration of adenosine-5′ phosphate by tenfold of that present in the standard reaction mixture had no inhibitory effect. Furthermore, since a sample of purified nicotinamide mononucleotide (free from adenosine-5′ phosphate), kindly supplied by Dr A. Kornberg, gave the same result as an equivalent

concentration of the nicotinamide mononucleotide normally employed, it may be concluded that the adenosine-5′ phosphate had no inhibitory effect on the reaction.

DPN destruction by mouse tissues

Table 1 shows that tissue dispersions of both mammary carcinoma and lactating gland destroy DPN, and that this activity is completely inhibited only by nicotinamide (0·7–0·8 M). On a nitrogen basis, the rate of destruction of DPN by tumour (approx. 4 μmoles of DPN/hr./mg. of N) somewhat exceeds that by normal lactating gland (2–3 μmoles of DPN/hr./mg. of N). Isolated nuclei from both tissues also destroyed DPN, the rates being generally similar (about 1 μmole of DPN/hr./mg. of N). This activity, both in tumour and gland nuclei, was completely inhibited by 0·7 M nicotinamide.

Table 1. *Inhibition of DPN destruction by dispersions of tumour tissue*

The reaction mixture (1·0 ml., pH 7·4) contained DPN (approx. 4×10^{-4} M), $MgCl_2$ (15×10^{-3} M), 0·3 ml. of glycyl-glycine buffer (0·25 M, pH 7·4), the inhibitor at the concentration shown and 0·1 ml. of dispersion of tumour tissue. Incubation was for 20 min. at 38°. The reaction was stopped by addition of 0·8 ml. of 20% (w/v) trichloroacetic acid and the DPN was estimated spectrophotometrically (Kornberg, 1950) at zero time and after 20 min.

Inhibitor	Inhibitor concn. (M)	Enzymic destruction of DPN (%)
None	—	94
Nicotinamide	0·2	55
	0·4	42
	0·6	7
	0·8	0
NaF	0·01	94
	0·06	95
Ammonium molybdate	0·01	70
$BeCl_2$	0·01	75

Comparison of mammary gland carcinoma with 'normal' (apparently non-malignant) mammary gland tissue from lactating and non-lactating (pregnant) mice

In Table 2 the results obtained with isolated nuclei from mammary tumour and normal gland tissue are compared on the basis of μmoles of DPN synthesized/hr./10^9 nuclei. It is seen that the mean value for nuclei from tumour tissue (group 3) is about one-fifth of that for nuclei from lactating mammary gland (group 1), and about one-third of that for nuclei from 'normal' non-lactating mammary gland from pregnant mice, whether tumour-bearing (group 2*a*) or not (group 2*b*). Comparison of groups 1, 2 and 3 in Table 2 shows that no single determination in any one group falls within the range of values of either of the other two groups.

Table 2 also shows that the differences between the mean values for tumour tissue and normal mammary-gland tissue (whether lactating or non-

Table 2. *Comparative rates of synthesis of DPN by nuclei from mouse mammary-gland and tumour tissue*

The assay procedure and other details are described in the text. In most cases, the nuclei for each determination were isolated from the pooled tissue taken from several mice. Assays were carried out at regular intervals during the period November 1954 to October 1955.

Tissue	Group	Strain of mice	No. of estimations	μmoles of DPN synthesized/hr./10^9 nuclei: Mean ± S.E.	Range
Lactating gland	1	*a* NZ	5	13·00 ± 1·34	10–17
		b C_3H	4	13·25 ± 1·25	10–16
'Normal' non-lactating gland	2	*a** C_3H	3	7·00 ± 0·56	6–8
		b† C_3H	1	8	—
Mammary carcinoma	3	C_3H	8	2·75 ± 0·15	2–3

Means of pooled values: Group 1 — 13·11 ± 0·87 (9); Group 2 — 7·25 ± 0·52 (4)

Ratios of mean values: $\frac{\text{Group 1}}{\text{Group 3}} = 4{\cdot}8$; $\frac{\text{Group 2}}{\text{Group 3}} = 2{\cdot}6$; $\frac{\text{Group 1}}{\text{Group 2}} = 1{\cdot}8$.

Statistical significance of differences between means:

Groups compared	Differences between mean values	*P*
1*a* and 1*b*	0·25	>0·9
1*b* and 3	10·50	<0·001
2*a* and 3	4·25	<0·001
1 and 2	5·86	<0·001

* Pregnant, tumour-bearing mice. † Pregnant, tumour-free mice.

Table 3. *Comparative rates of synthesis of DPN by nuclei from livers of mice in different age groups*

Nuclei were isolated by the same method as used for mammary gland (see Results). The assay procedure is described in the text. The livers of litter mates (both sexes) were pooled to obtain sufficient material for isolation of nuclei from foetal and young mice. Adults (over 3 months old) were all females except for one male NZ mouse.

Group	Age	Strain of mice	No. of estimations	μmoles of DPN synthesized/ hr./10^9 nuclei
A	Foetal	C_3H	2	2
		*	1	3
B	7 days	C_3H	1	12
	17 days	C_3H	1	13
		NZ	1	11
C	Adult	C_3H	2	42
		NZ	4	45

Mean values for pooled livers: Group *A* 2·3±0·3 (3); Group *B* 12·0±0·6 (3); Group *C* 44·0±4·2 (6)

Ratios of mean values: $\frac{\text{Group } B}{\text{Group } A}=5{\cdot}2$; $\frac{\text{Group } C}{\text{Group } A}=19{\cdot}1$; $\frac{\text{Group } C}{\text{Group } B}=3{\cdot}7$.

Statistical significance of differences between means:

Groups compared	Differences between mean values	*P*
A and *B*	9·7	<0·001
A and *C*	41·7	<0·001
B and *C*	32·0	<0·001

* Laboratory strain (not a pure-bred line) kindly supplied by Walter and Eliza Hall Research Institute.

lactating) are statistically highly significant. The difference between the mean values for lactating and non-lactating gland tissue is also statistically highly significant. However, there is no statistical difference between the mean values for the nuclei from lactating glands of C_3H and NZ mice (Table 2).

Comparison of livers of foetal, very young (7–17 days old) and adult mice (over 3 months old)

Table 3 shows the comparison of liver tissue from foetal (group *A*), very young (group *B*) and adult (group *C*) mice on the basis of μmoles of DPN synthesized/hr./10^9 nuclei. The mean value for nuclei from foetal mice is extremely low, while that from young mice is about one-quarter of that for nuclei from adult mice. The difference between the mean values for each group is statistically highly significant (Table 3).

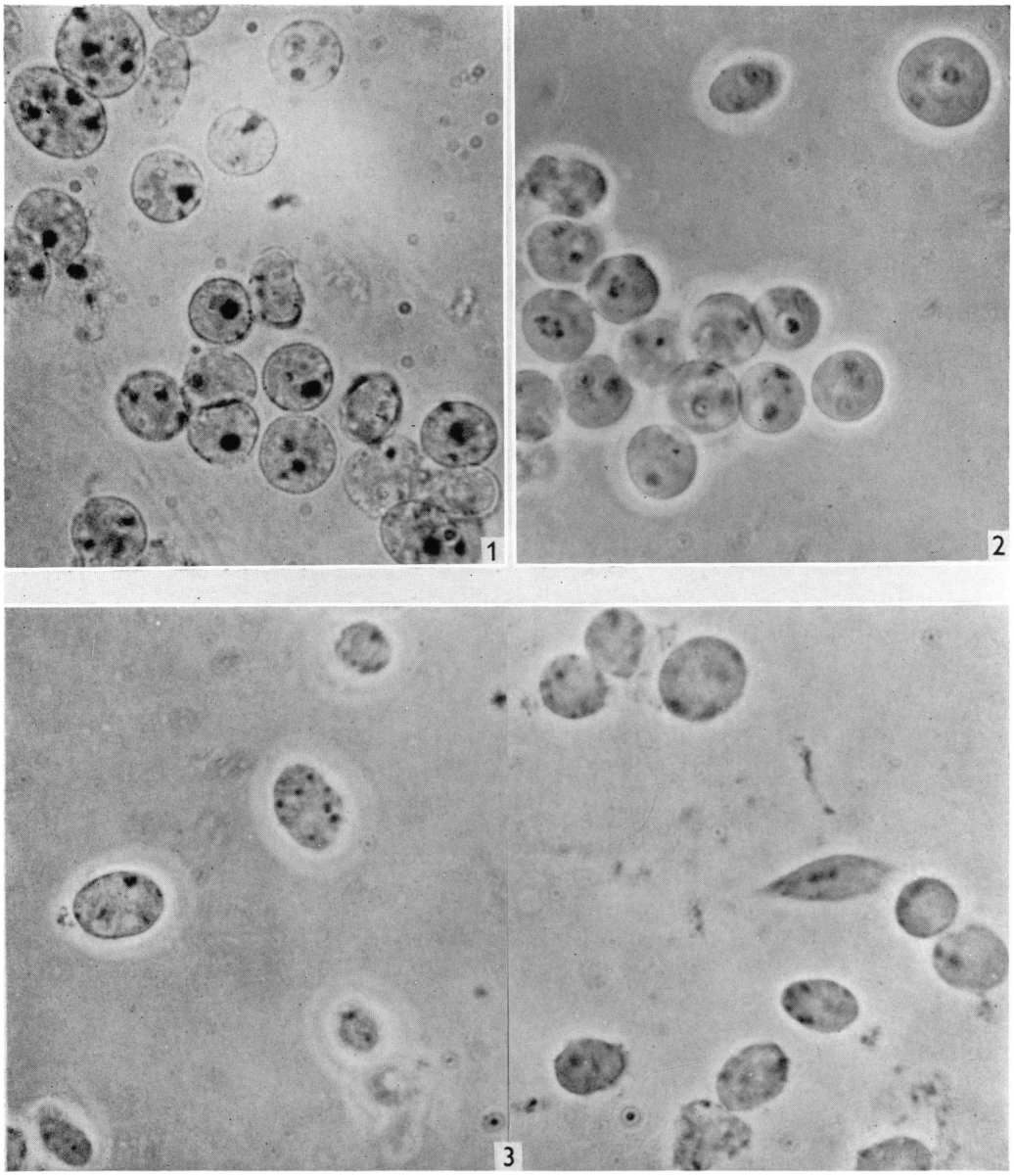
The assays for the livers are based on counts which included nuclei of all sizes. It is not known whether the rate of synthesis varies with the size of the nucleus.

DISCUSSION

Of the several ways in which the metabolic activities of normal and tumour tissue may be compared, it appears to us that the most valid is the relative activity/whole cell (see also Fishman, 1953). An alternative basis for comparison, which is probably equally valid, is the relative activity/nucleus, as used in the present investigation. In general, the preparations of nuclei from all tissues were satisfactory in showing very few whole cells. A small amount of particulate material could be observed in most preparations, even after repeated washing by relayering and centrifuging, but most of the soluble constituents of the cytoplasm would have been removed in the washing treatment. It is therefore considered that, while slight contamination with particulate material would have affected the results when expressed on a dry weight or nitrogen basis, it has negligible effect on the basis of counts of nuclei.

Photomicrographs of nuclei isolated from normal lactating mammary gland (Pl. 2, 1 and 2) and from mammary carcinoma (Pl. 2, 3) are shown in the accompanying plate. Tumour nuclei in the same suspending fluid as used for mammary gland nuclei (Pl. 2, 1) would not take up the methylene blue sufficiently to permit satisfactory photography by direct lighting. Although there is a good deal of variation in the size of liver nuclei, those isolated from embryo mice were generally smaller, and those from adult mice generally larger, than those from livers of young mice. The nuclei from the latter group appeared to be about the size of those from mammary gland and tumour.

Marked changes have been observed in the activities (expressed on the basis of tissue weight) of some cytoplasmic enzymes of mammary gland during pregnancy and lactation (see, for example,



Photomicrographs of nuclei isolated from mouse tissues and suspended in 0·25 M sucrose–0·0009 M-$CaCl_2$ solution. 1 and 2: nuclei from 'normal' lactating mammary gland from strain C_3H mice, stained with methylene blue (1), and unstained but taken by phase contrast (2). 3: nuclei from mammary-gland carcinoma from strain C_3H mice, taken by phase contrast. Nuclei as shown × 1350.

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(*Facing p.* 644)

Folley & Greenbaum, 1947; Moore & Nelson, 1952). It would therefore have been desirable to obtain activities for nuclei from mammary glands from non-pregnant mice and from animals in early pregnancy. Unfortunately, insufficient tissue for assay by the present technique could be obtained from such females (see under Results). Table 2 shows, however, that the DPN-pyrophosphorylase activity/nucleus in a lactating gland is about 1·8 times that in non-lactating tissue, and that this difference is significant.

Cell division is generally very rapid in tumour tissue. Nuclei from a mammary tumour have a markedly lower activity for synthesis of DPN than nuclei from either lactating or non-lactating glands (Table 2). Moreover, nuclei from the very rapidly growing embryonic-liver tissue of foetal mice, and from normal livers from young mice, have a much lower activity than nuclei from livers of adult animals (Table 3). The very low value for the nuclei from embryonic tissue (Table 3) is particularly noteworthy. These results suggest that a decreased rate of synthesis of DPN may be one aspect of rapid cell proliferation, so greatly accentuated in a tumour. It would be of interest to determine whether such rapid cell division would be reduced if the rate of supply of DPN could be increased by some means.

The results given earlier (Table 1 and p. 643) clearly establish that both the tumour and mammary gland tissue rapidly break down DPN *in vitro*. This destruction of DPN may be due to the action of several different enzymes (see review by Singer & Kearney, 1954). However, the inhibition by nicotinamide suggests that most of the breakdown is due to the action of DPN nucleosidase (see Mann & Quastel, 1941). In tissue dispersions of rat liver, this enzyme appears to be localized mainly in the 'microsome' fraction obtained from the cytoplasm (Sung & Williams, 1952). However, Sung & Williams (1952) observed that a relatively large percentage of the DPN nucleosidase activity was associated with the nuclear fraction. Washed nuclei from both mammary gland and tumour also break down DPN (p. 643). This may be due to the activity of the modified elements of the endoplasmic reticulum associated with the nucleus, which Watson (1955) has called the *perinuclear cisternae*. The higher concentration of nicotinamide (0·7 M) used in the assay of DPN synthesis (p. 641), as compared with the much lower (0·2 M) concentration used by Hogeboom & Schneider (1952), was necessary in order to inhibit completely the breakdown of DPN by the mammary-gland and tumour tissue. There was no evidence of inhibition of DPN synthesis by this relatively high concentration of nicotinamide.

When this work was well advanced, Jedeikin & Weinhouse (1955) made a preliminary report of their finding that the total DPN contents of a variety of transplanted solid and ascites tumours are considerably less than those of most normal tissues. They also observed a lowered total DPN content in hepatoma induced in rats by prolonged administration of 4-dimethylaminoazobenzene, as compared with non-neoplastic liver from the same animals. It seems unlikely that this lowered DPN content of tumours could be due to increased destruction of this coenzyme. With inhibition of yeast fermentation as a measure of DPN-ase activity, Quastel & Zatman (1953) observed that the activity of a variety of tumours was in the range of, and frequently less than, that of most normal tissues. Moreover, the DPN-ase activity of hepatoma did not differ significantly from that of the control tissue. Hence it seems very probable that the results obtained by Jedeikin & Weinhouse (1955) are due to a decreased DPN-pyrophosphorylase activity in the neoplastic tissues, as found in the present studies with mammary tumour. This may well be a general feature of malignant tissues.

A large number of observations have suggested that an enzymic lesion connected with DPN-linked systems may be a significant feature of metabolism of tumour as compared with normal tissue. Warburg (1928) and Dickens & Weil-Malherbe (1943) observed a much higher aerobic glycolysis in slices of tumour than in normal tissues. However, when dispersions of tumour tissue (or the appropriate cytoplasmic fractions) are adequately supplemented with DPN (for which the requirement is higher than for normal tissues), then both glycolysis of hexose diphosphate (LePage, 1948, 1950) and oxidation of pyruvate (Wenner, Spirtes & Weinhouse, 1951; Wenner & Weinhouse, 1953) proceed at rates which differ little from those of comparable normal tissues. Moreover, Kensler, Suguira & Rhoads (1940) observed that the DPN level of rat liver falls during administration of 4-dimethylaminoazobenzene, an active liver carcinogen. It is in the light of these observations that the significance of the decreased rate of synthesis of DPN by nuclei from mammary-gland carcinoma, as compared with normal gland (Table 2), must be assessed. The kinetic consequences of a restricted supply of DPN to enzyme systems which will compete for this common cofactor are by no means clear, but possible effects can be seen from considerations of the kind discussed by Dixon (1951), and recently extended by Racker (1955), in relation to interaction of co-enzyme-linked enzyme systems. As suggested in the introduction, there may be profound disturbance of normal cytoplasmic reactions.

In a valuable review which appeared after this paper was submitted for publication, Weinhouse (1955) has discussed the 'DPN effect' in relation to tumour metabolism. While he makes no comment

on the possible impairment of nuclear DPN-pyrophosphorylase activity in tumours, Weinhouse (1955) has also drawn attention to the sensitive control over metabolic behaviour of the cell which may be exercised through the rate of supply of enzymic cofactors.

The investigation is being extended to determine the factors which may cause the changed activity of the DPN pyrophosphorylase.

SUMMARY

1. Nuclei were isolated from lactating and non-lactating mammary glands from strains of mice with high (C_3H) and low (NZ) tumour incidence, and from mammary-gland carcinomata from the C_3H mice.

2. Tissues were compared on the basis of the rates of synthesis of diphosphopyridine nucleotide from adenosine triphosphate and nicotinamide mononucleotide by the isolated nuclei. The mean rates of synthesis (per 10^9 nuclei) by tumour was about one-third of that of gland from pregnant, non-lactating mice and about one-fifth of that of lactating gland tissue. The results were very consistent, and in all cases the differences between means were statistically highly significant.

3. There was no statistical difference between the mean rates for lactating glands from C_3H and NZ mice.

4. The mean rates of synthesis (per 10^9 nuclei) were determined for nuclei isolated from livers of foetal, young and adult mice. The activities for the foetal and young mice were very much less than that of the adult mice.

5. The significance of these findings is discussed in the light of previous work relating to a possible lesion in tumour tissue involving enzyme systems linked with diphosphopyridine nucleotide.

We wish to thank Dr A. Haddow, of the Chester Beatty Cancer Research Institute, London, and Dr F. Bielschowsky, of the Medical Research Institute, University of Otago, Dunedin, New Zealand, for supplying the strains of mice used in this investigation. We are much indebted to Dr G. Christie, of the Department of Pathology, for helpful advice and particularly for histological examination of tumours. The photographs of isolated nuclei were taken by Mr E. Matthaei, to whom we wish to express our thanks.

This investigation was aided by a grant from the Anti-Cancer Council of Victoria, whose assistance is gratefully acknowledged.

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