

## Partial Purification, Properties and Regulation of Inosine 5'-Phosphate Dehydrogenase in Normal and Malignant Rat Tissues

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IMP dehydrogenase (EC 1.2.1.14) was purified 180-fold from rat liver and from the transplantable rat hepatoma 3924A. The enzymes from the two sources were apparently identical; they exhibited hyperbolic saturation kinetics and an ordered, sequential mechanism, and were subject to inhibition by a number of purine nucleotides.  $K_m$  values for the substrates, IMP and  $NAD^+$ , were 12 and 24  $\mu M$  respectively. IMP dehydrogenase activity in a spectrum of rat hepatomas was increased, relative to normal liver, by 2.5-13-fold; these increases correlated with tumour growth rate. Activity in two rat kidney tumours was increased 3-fold relative to that in normal renal cortex; control of activity of this enzyme is apparently altered in neoplastic cells. After partial hepatectomy, IMP dehydrogenase activity began to rise 6 h after operation, reaching a peak of 580% of normal activity by 18 h. Activity in neonatal liver, however, was only slightly higher than that in the adult. Organ-distribution studies showed highest enzyme activities in spleen and thymus. In livers of rats starved for 3 days, where all enzymes, except those involved in gluconeogenesis, showed decreased activity, IMP dehydrogenase activity was increased; this change was accompanied by a rise in hepatic GTP concentrations. It is concluded that IMP dehydrogenase is a key enzyme in the regulation of GTP production, and thus involved in regulation of nucleic acid biosynthesis. The increased activity of IMP dehydrogenase in liver of starved rats may be related to the requirement for GTP for gluconeogenesis.

IMP occupies a central position in purine metabolism, acting as the common precursor for nucleotides of adenine and guanine, and also leading to the purine degradative pathway. Short-term regulation of purine interconversions is achieved by a combination of positive and negative feedback effects (Hartman, 1970). However, when a cell makes extensive changes in its pattern of metabolic activity, as during differentiation, tissue regeneration, in response to hormone stimuli or after malignant transformation, the adjustments to regulation are frequently effected by an alteration in gene expression, rather than by inhibition or activation of existing enzyme molecules (Weber, 1974). Few studies have been reported about IMP dehydrogenase under such conditions.

Previous studies showed increased activity of the first enzyme of purine biosynthesis *de novo*, glutamine phosphoribosyltransferase (amidophosphoribosyltransferase; EC 2.4.2.14) in normal and malignant proliferating cells (Prajda *et al.*, 1975) and a decrease in the xanthine oxidase activity of tumours (Reid & Lewin, 1957; De Lamirande *et al.*, 1958; Prajda *et al.*, 1976). These

observations directed our attention to the metabolic fate of IMP. The initial reaction of the guanine nucleotide branch, the oxidation of IMP to XMP, with concomitant reduction of  $NAD^+$ , is catalysed by IMP dehydrogenase (EC 1.2.1.14) (Magasanik *et al.*, 1957; Abrams & Bentley, 1955; Lagerkvist, 1958). This enzyme has been assayed previously in several mammalian tissues, and examined in some detail in mouse sarcoma 180 cells (Anderson & Sartorelli, 1968) and human placenta (Holmes *et al.*, 1974). These mammalian enzymes were subject to feedback inhibition by GMP, in competition with IMP. In addition to being inhibited by natural nucleotides, IMP dehydrogenase was a target enzyme for a number of anti-tumour drugs, including 6-thioinosinic acid, mycophenolic acid and 6-chloropurine ribonucleotide (Atkinson *et al.*, 1963; Sweeney *et al.*, 1972; Anderson & Sartorelli, 1969). We have recently reported (Jackson *et al.*, 1975) increased IMP dehydrogenase activity in conditions of normal and malignant cell proliferation. The present paper describes new results about partial purification, properties and comparative studies of this enzyme in developing and regenerating liver, and in trans-

plantable hepatomas and kidney tumours of the rat. The role of this enzyme in gluconeogenesis has also been investigated and the concentrations of GTP, ATP, UTP, CTP and ITP were determined in freeze-clamp preparations.

## Experimental

### *Animals and tumours*

Hepatomas 3924A and 3683 were carried in ACI/N rats, all other tumours in Buffalo-strain rats. Tumours were implanted subcutaneously bilaterally and harvested at a diameter of about 1.5 cm. The biological properties of these tumours and methods of tissue preparation have been reported previously (Ferdinandus *et al.*, 1971; Morris & Wagner, 1968). For regenerating liver studies, ACI/N rats of 200 g body wt. were used. Partial hepatectomy was carried out under light diethyl ether anaesthesia by the standard method (Higgins & Anderson, 1931). Sham-operated animals were used as controls; the operations were timed such that all animals were killed between 09:00 h and 10:00 h. In experiments with the differentiating liver of rats under 7 days old, three or more samples were prepared, each containing pooled livers of three to six animals; beyond this age individual livers were used. Liver and tumour cells were assessed by visual counting of orcein-stained nuclei (Weber & Cantero, 1957). In experiments with blood cells, erythrocytes and leucocytes were separated by dextran sedimentation (Skoog & Beck, 1956), and the leucocyte pellet was freed from residual erythrocytes by osmotic shock (Walford *et al.*, 1957).

### *IMP dehydrogenase assays*

The standard enzyme assay used 0.1 M-potassium phosphate buffer, pH 7.4, containing 1 mM-EDTA and 0.5 mM-mercaptoethanol; NAD<sup>+</sup> was used at a final concentration of 0.2 mM, the mixture was equilibrated with enzyme, and the reaction was started by the addition of IMP to a final concentration of 0.17 mM. As a routine 0.2 ml of enzyme preparation (containing about 0.4 mg of protein) was used in a total volume of 1.3 ml, in a 10 mm-path-length semi-cuvette. The reaction was monitored at 37°C in a Cary 118CX spectrophotometer, against a reference cuvette containing no IMP. The wavelength used was normally 290 nm but in some studies was 340 nm; rates were calculated by assuming  $\Delta A_{\text{nm}}$  for the reaction of  $5.4 \text{ cm}^{-1}$  (290 nm) or  $6.2 \text{ cm}^{-1}$  (340 nm). In crude homogenates, an NAD<sup>+</sup>-independent increase occurred in the  $A_{290}$ ; this effect increased with time, and was attributed to combined action of 5'-nucleotidase, purine nucleoside phosphorylase and xanthine oxidase.

Radioactive assays used the same reaction mixture

as the optical assay, but with the addition of  $0.4 \mu\text{Ci}$  of [<sup>3</sup>H]IMP, in a total volume of 0.25 ml. The reaction was stopped by the addition of 0.02 ml of 2.6 M-HClO<sub>4</sub>; after centrifugation the supernatant was neutralized by the addition of 0.02 ml of 2.7 M-KOH. A sample (10  $\mu\text{l}$ ) of the neutralized reaction mixture was applied as a 1 cm streak to the origin of a PEI (phosphoethyleneimine) cellulose t.l.c. plate (previously washed in 0.5 M-sodium formate, pH 3.4, followed by aq. 50%, v/v, methanol). A mixed carrier solution (10  $\mu\text{l}$ ) containing AMP, IMP, XMP, GMP, ADP and adenylosuccinate, all at 10 mM, was applied to the plate, over the experimental samples. Plates were developed in 0.5 M-sodium formate buffer, pH 3.4, for 2.5 h. The carrier spots gave the following  $R_F$  values: AMP, 0.74; IMP, 0.51; XMP, 0.38; GMP, 0.26; ADP, 0.12; adenylosuccinate, 0.06. Nucleosides and bases migrated at the solvent front, and ATP, GTP and GDP remained at the origin. The portions of the chromatogram containing the various compounds were cut out and counted for radioactivity in a scintillation counter (with Aquasol; New England Nuclear, Boston, MA, U.S.A.). The 5'-nucleotidase activity was assayed in the same system by measuring the conversion of [<sup>3</sup>H]IMP into inosine. Protein was determined by the biuret reaction with crystalline bovine plasma albumin as standard (Gornall *et al.*, 1949).

### *Assay of nucleoside triphosphate concentrations in liver*

Hepatic concentrations of nucleoside triphosphates were determined by the freeze-clamp method of Williamson *et al.* (1967). The preparation of the samples and the measurements of concentrations of ATP, GTP, UTP, CTP and ITP by high-pressure liquid chromatography were carried out as described by Jackson *et al.* (1976).

### *Chemicals*

Substrates, cofactors and thyroid hormones were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. [<sup>8-3</sup>H]IMP (sp. radioactivity 5.5 Ci/mmol) was purchased from Amersham-Searle, Arlington Heights, IL, U.S.A. It was further purified by t.l.c. on PEI-cellulose; a sample containing about 0.2 mCi was applied to the plate (which had been washed before use with 0.1 M-NaCl) and the plate was then developed for 15 min in 1 M-LiCl. The chromatogram was dried and washed three times in water. The IMP-containing region was identified under a u.v. lamp, cut out and eluted with aq. 2% NH<sub>3</sub>. The material was freeze-dried and before use diluted with non-radioactive IMP to a concentration of 50 mM and a specific radioactivity of 0.4 mCi/mmol. The thin-layer plates were a product of Macherey-Nagel and Co., Düren, Germany. Other reagents were from local suppliers and analytical grades were used when available.

## Results

### Enzyme purification

About 30 g (wet wt.) of liver was homogenized for 30 s at 600 rev./min in a Thomas tissue grinder, in 120 ml of 0.25 M-sucrose/10 mM-Tris/HCl, pH 7.4. The homogenate was centrifuged for 90 min at 105 000 g. The supernatant fraction was treated with  $(\text{NH}_4)_2\text{SO}_4$  and the portion precipitating between 30 and 40% saturation contained all the IMP dehydrogenase activity. It was dissolved and dialysed against 200 vol. of assay buffer (0.1 M-potassium phosphate, pH 7.4/1 mM-EDTA/0.5 mM-2-mercaptoethanol). The dialysed enzyme preparation was then applied to a column (15 cm  $\times$  1 cm<sup>2</sup>) of Sephadex A-50, and eluted with 50 mM-Tris/HCl, pH 7.4/0.5 mM-mercaptoethanol. The active fractions were concentrated by vacuum dialysis and applied to a column (45 cm  $\times$  1.8 cm<sup>2</sup>) of Sephadex G-150, which was eluted with the same buffer. After reconcentration, samples were chromatographed on a column (20 cm  $\times$  1 cm) of Whatman DEAE-cellulose (DE 52) with the same gradient as used for stage 3.

Results are summarized in Table 1. Routine assays in tissue samples used the dialysed 30–40% satd.  $(\text{NH}_4)_2\text{SO}_4$  precipitate (stage 2 in Table 1). The enzyme extract at this stage of purification had low nucleotidase activity (conversion of IMP into inosine less than 10 nmol/h per mg of protein). However, in the crude cytosol fraction, interfering activity was considerable, and the estimate of IMP dehydrogenase activity was low, leading to an apparent recovery of over 200% at stage 2. The true recovery was estimated by the addition of known amounts of purified enzyme to the cytosol; this study indicated that losses through the first two stages of purification were less than 30%. The purest liver IMP dehydrogenase preparation obtained had a specific activity of 0.00649 i.u./mg of protein, at 37°C. Enzyme from rapidly growing hepatoma 3924A was purified by a similar procedure; specific activity at each stage was approximately ten times that for the liver enzyme at a corresponding stage of purification. The most active hepatoma 3924A IMP dehydrogenase obtained had a

final specific activity of 0.0775 i.u./mg of protein (at 37°C), representing a purification of 188-fold.

### Effect of dialysis

After resuspension of the 40% satd.  $(\text{NH}_4)_2\text{SO}_4$  precipitate (stage 2), dialysis for 2 h (against 500 vol. of assay buffer at 0°C) resulted in over 2-fold activation as residual  $(\text{NH}_4)_2\text{SO}_4$  was removed, for both liver and hepatoma preparations. Dialysis for a further 2 h in each case increased activity by a further 10%. Thereafter, further dialysis (at 4°C) resulted in loss of activity at the rate of about 3%/h. This loss of activity appeared to be permanent: it was not reversed by re-addition of the diffusate (up to 50 vol.) or by dithioerythritol up to 2 mM.

### Characterization of reaction products

The radioactive assay described above was used to identify the products of the enzyme-catalysed reaction. After incubation with the complete system, with dialysed stage-2 enzyme, thin-layer chromatograms were run and treated as described in the 'Experimental' section. Radioactivity was associated with the XMP position of the chromatogram. No radioactivity was observed in the GMP, AMP or SAMP positions, or at the solvent front (i.e. with purine nucleosides and bases) or at the origin. If enzyme was omitted from the system, or if boiled enzyme was substituted, all the radioactivity was recovered in the IMP position. When enzyme was present, the amount of <sup>3</sup>H converted into XMP was proportional to time (up to 45 min) and to the amount of enzyme (up to 0.001 i.u.). When NAD<sup>+</sup> was omitted from the reaction mixture, no radioactivity was detected in XMP. These experiments provided satisfactory evidence of the identity of the reaction under study, and of the lack of interfering activities. The optical assay was used to check the stoichiometry of the reaction. Reduction of NAD<sup>+</sup> to NADH was first measured at 340 nm. The reaction was then monitored at 290 nm, and the  $\Delta A$  resulting from NAD<sup>+</sup> reduction (assuming a  $\Delta A_{\text{mM}}$  of 0.7 cm<sup>-1</sup> at this wavelength) was subtracted from the total  $\Delta A_{290}$ . The corrected  $\Delta A_{290}$  was used to calculate the

Table 1. *Partial purification of IMP dehydrogenase from rat liver*

Purification was calculated by assuming that the total activity at stage 1 is the same as that at stage 2. Apparent recovery is given as a percentage relative to stage 2.

Stage of purification	Total activity (i.u.)	Total protein (mg)	Specific activity (i.u./g)	Purification (fold)	Apparent recovery (%)
1. 105 000 g (90 min) supernatant	0.042	2670.0	0.016		
2. 30–40% $(\text{NH}_4)_2\text{SO}_4$ fraction, then 4 h dialysis	0.099	162.9	0.61	16	100
3. Sephadex A-50 ion-exchange chromatography	0.103	60.3	1.7	46	104
4. Sephadex G-150 gel filtration	0.067	16.7	4.0	108	68
5. DE-52 chromatography	0.048	7.4	6.49	174	49

XMP produced, assuming a  $\Delta A_{\text{nm}}$  from IMP to XMP of  $4.7\text{cm}^{-1}$  (Atkinson *et al.*, 1963). For most stage-2 IMP dehydrogenase preparations the ratio NADH formed/XMP formed was about 0.96. For crude preparations the ratio was always lower, often between 0.6 and 0.8, indicating re-oxidation of the NADH formed.

For routine assay purposes the optical method, as described in the 'Experimental' section, was used. Under the conditions described, rates were linear with time for at least 30 min, and proportional to enzyme concentrations up to  $0.0015\text{ i.u./ml}$ .

#### *Kinetic properties of the purified IMP dehydrogenase*

Liver and hepatoma 3924A IMP dehydrogenases exhibited a pH optimum at about pH 8.1 (Fig. 1); all subsequent work was conducted at pH 7.4, where the activity was about 80% of the optimum value. The enzyme required a univalent cation for activity,  $\text{K}^+$  being the most effective. The apparent  $K_m$  for  $\text{K}^+$  was determined (Table 2), but the kinetics of  $\text{K}^+$  binding were not studied further. Recoveries of enzyme activity during the purification procedure were improved if EDTA was present, and a concentration of 1 mM was normally used. Thiols caused a biphasic effect, stabilizing the enzyme at low concentration, but inhibiting at higher concentrations (above 1 mM for dithioerythritol, above 2 mM for mercaptoethanol). The buffers used for purification and assay contained 0.5 mM-mercaptoethanol. Substrate-saturation curves for [IMP] ( $0.2\text{ mM-NAD}^+$ ) and for  $[\text{NAD}^+]$  (at  $0.17\text{ mM-IMP}$ ) are shown in Fig. 2. The excessive substrate inhibition caused by

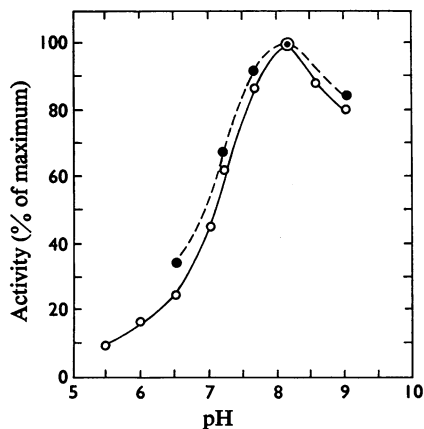


Fig. 1. *pH-activity profiles for IMP dehydrogenases from rat liver and hepatoma 3924A*

●, Liver enzyme; ○, hepatoma enzyme. For pH 7 and below, 0.1 M-potassium phosphate was used and for pH 7 and above, 0.05 M-Tris/HCl/0.1 M-KCl. Both buffers were used at pH 7, and gave identical results.

$\text{NAD}^+$  is discussed below. Subsequent initial-velocity and product-inhibition studies used  $\text{NAD}^+$  concentrations not greater than 0.25 mM. Apparent  $K_m$  values for each substrate depended on the concentration of the other substrate. As concentrations of the fixed substrate were decreased, the apparent  $K_m$  for the varied substrate increased, ruling out the 'ping-pong' mechanism (Cleland, 1963). Apparent  $K_m$  values were computed directly from initial-velocity determinations by non-linear regression (Wilkinson, 1961). Results are shown in double-reciprocal graphical form in Fig. 3, and limiting  $K_m$  values are tabulated in Table 2. Fig. 4 shows the results of product-inhibition studies; again, parameters were calculated by hyperbolic regression, but are shown as double-reciprocal plots. XMP was competitive with IMP and non-competitive with  $\text{NAD}^+$ , and NADH was non-competitive with both IMP and  $\text{NAD}^+$ . The initial-velocity and product-inhibition data suggest an ordered, sequential mechanism in which IMP binds to free enzyme, followed by  $\text{NAD}^+$ , then, after re-arrangement of the ternary complex, NADH is released and finally XMP is released (Cleland, 1963). The excessive substrate inhibition caused by  $\text{NAD}^+$  was investigated further, and proved to be non-competitive with IMP. Anderson & Sartorelli (1968) demonstrated a similar effect with the sarcoma 180 enzyme, and showed that this inhibition was due to formation of a 'dead end' ternary complex (XMP-enzyme- $\text{NAD}^+$ ). Our data support a similar explanation of the present results. The total  $\text{NAD}^+$  concentration in rat liver was reported to be 0.67 mM (Burch *et al.*, 1963). Fig 2 shows that this  $\text{NAD}^+$  concentration would result in 50% inhibition of IMP dehydrogenase. The published value refers to total  $\text{NAD}^+$ ; if bound  $\text{NAD}^+$  forms an appreciable proportion of the total, then the inhibition of IMP dehydrogenase,

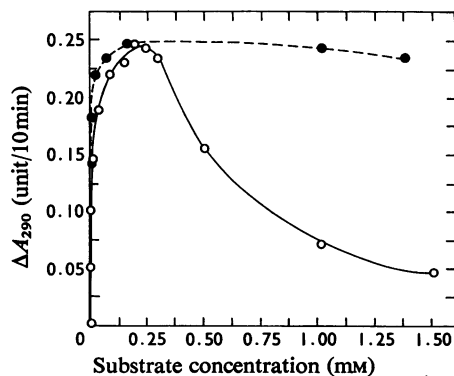


Fig. 2. *Substrate saturation curves for rat liver purified IMP dehydrogenase*

○,  $\text{NAD}^+$  saturation curve; ●, IMP saturation curve.

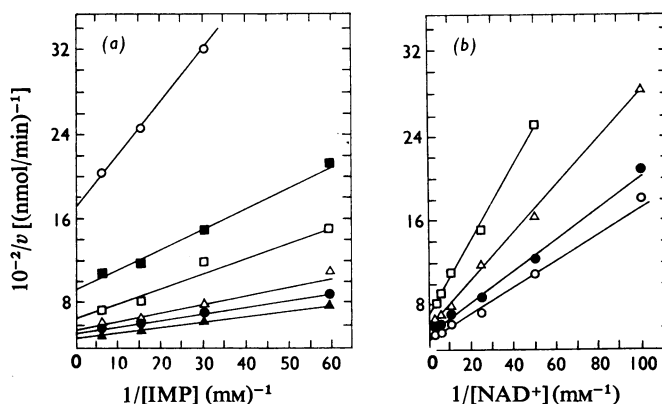


Fig. 3. Double-reciprocal plots of initial velocity data for purified liver enzyme (a) Variable substrate, IMP:  $\circ$ ,  $10\ \mu\text{M-NAD}^+$ ;  $\blacksquare$ ,  $20\ \mu\text{M-NAD}^+$ ;  $\square$ ,  $40\ \mu\text{M-NAD}^+$ ;  $\triangle$ ,  $100\ \mu\text{M-NAD}^+$ ;  $\bullet$ ,  $200\ \mu\text{M-NAD}^+$ ;  $\blacktriangle$ ,  $250\ \mu\text{M-NAD}^+$ . (b) Variable substrate,  $\text{NAD}^+$ :  $\square$ ,  $17\ \mu\text{M-IMP}$ ;  $\triangle$ ,  $34\ \mu\text{M-IMP}$ ;  $\bullet$ ,  $68\ \mu\text{M-IMP}$ ;  $\circ$ ,  $170\ \mu\text{M-IMP}$ . General assay conditions were as described in the Experimental section.

Table 2. Comparison of kinetic properties of mammalian IMP dehydrogenases

The terminology follows that of Cleland (1963) and all values are given as  $\mu\text{M}$ . In addition to the data for rat liver enzyme determined in the present study ( $\pm$ s.e. where available) literature values for the human placenta and mouse sarcoma enzymes are included for comparison.  $I_{0.5}$  values refer to standard assay conditions and  $I_{50}$  is the concentration of inhibitor required to produce 50% inhibition under standard assay conditions.

Parameters	Rat liver purified enzyme (present study)	Human placenta (Holmes <i>et al.</i> , 1974)	Mouse sarcoma 180 (Anderson & Sartorelli, 1968)
$K_a$ for IMP	$11.6 \pm 2.5$	14	16
$K_{1a}$ for IMP	$34.6 \pm 4.6$		
$K_b$ for $\text{NAD}^+$	$24.3 \pm 3.8$	46	46
$K_1$ for XMP	$25.9 \pm 3.1$	30	
$K_1$ (slope) for NADH	$360 \pm 66$		
$K_1$ (intercept) for NADH	$260 \pm 10$		
$K_1$ for GMP	$88 \pm 12$	100	
$K_1$ for AMP	$650 \pm 45$	530	
$K_{m, \text{app}}$ for $\text{K}^+$	$7900 \pm 400$	17000	2000
$I_{50}$ for excess of $\text{NAD}^+$	650		2000
$I_{50}$ for NADPH	138		<100
pH optimum	8.1		8.0
$I_{0.5}$ for mycophenolic acid	400		
$I_{0.5}$ for 6-thio-IMP	31		

determined by the free  $\text{NAD}^+$  concentration, might be considerably less. Also, the published value was determined for whole liver; the cytosol concentration may differ.

GMP inhibited the liver and hepatoma IMP dehydrogenases and this inhibition was competitive with IMP; AMP had a similar but weaker effect (Table 2). The ratio of guanine nucleotides/adenine

nucleotides may therefore play a controlling role at this metabolic branch point. The IMP dehydrogenase of sarcoma 180 was reported in a previous study (Anderson & Sartorelli, 1968) to be susceptible to NADPH inhibition. Our experiments confirmed that this was the case with the rat liver enzyme; under the usual assay conditions 50% inhibition was given by  $0.14\ \text{mM-NADPH}$ .

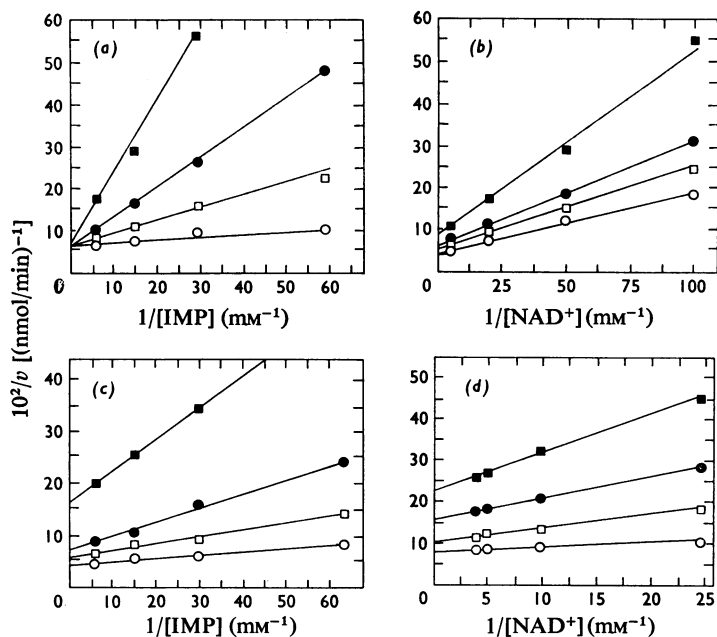


Fig. 4. Product-inhibition studies of purified liver enzyme

(a) Inhibition by XMP, variable substrate IMP: ■, 600  $\mu\text{M}$ -XMP; ●, 200  $\mu\text{M}$ -XMP; □, 80  $\mu\text{M}$ -XMP; ○, no XMP.  $\text{NAD}^+$  at 200  $\mu\text{M}$  in each case. (b) Inhibition by XMP, variable substrate  $\text{NAD}^+$ ; XMP concentrations as for (a). IMP at 170  $\mu\text{M}$  in each case. (c) Inhibition by NADH, variable substrate IMP; ■, 500  $\mu\text{M}$ -NADH; ●, 200  $\mu\text{M}$ -NADH; □, 100  $\mu\text{M}$ -NADH; ○, no NADH.  $\text{NAD}^+$  at 200  $\mu\text{M}$  in each case. (d) Inhibition by NADH, variable substrate  $\text{NAD}^+$ . ■, 350  $\mu\text{M}$ -NADH; ●, □ and ○ as for (c). IMP at 170  $\mu\text{M}$ . General assay conditions were as described in the Experimental section.

#### Experiments with thyroid hormones

Al-Mudhaffar & Ackerman (1968), in a study of rat liver IMP dehydrogenase, claimed that the enzyme was inhibited by L-tri-iodothyronine and L-tetra-iodothyronine (thyroxine) at low concentrations. We have been unable to repeat these observations with our enzyme preparation. The main difference in experimental conditions between our experiments and those of the previous investigators was that our assay buffer was at pH 7.4 instead of pH 8.3. We used stage-3 enzyme (DEAE-Sephadex-treated) and used the thyroid hormones at concentrations bracketing the reported maximally effective concentration (0.5, 1.0 and 10 nM for L-tri-iodothyronine and 0.032, 0.13 and 1.3  $\mu\text{M}$  for L-tetra-iodothyronine). All rates were within 4% of control values. Pre-incubation of enzyme with the hormones for periods up to 45 min did not produce any measurable change in activity relative to the controls.

#### Distribution of IMP dehydrogenase in rat tissues

The enzyme was measured in a variety of rat organs; in all cases the tissue was homogenized in 0.25M-sucrose, and the material purified by the same method used for liver, to the end of stage 2. The

partially purified material was free from interfering activity in every case except that of kidney. The renal extracts showed large, IMP-independent absorbance changes at both 340 and 290nm; it was, however, possible to estimate IMP dehydrogenase activity if blank cuvettes without IMP were read simultaneously and if reaction times did not exceed 5 min. IMP dehydrogenase activities are summarized in Table 3. A correlation with cell proliferation is clearly evident. Thymus and spleen had the highest activities of the tissues examined, and bone marrow and testis also showed greater activity than liver. Small intestinal epithelium proved an exception to this trend. Three separate layers were analysed, but the activities in all were comparatively low.

#### Regenerating liver

The present experiments extended previous studies on IMP dehydrogenase in this system to include the early events in hepatic regeneration. A significant increase in activity was already apparent 6h after the operation, and by 18h a peak of almost 6-fold the normal value was reached. Activities at this time were as follows (means  $\pm$  S.E.M. of five animals in each group): sham-operated  $2.2 \pm 0.2$  nmol/h per mg of

Table 3. *Distribution of IMP dehydrogenase in various organs*

Experimental details are given in the text. Values shown are means of triplicate samples, except for bone marrow, erythrocytes and leucocytes, where pooled samples from three or more rats were used.

Tissues	Soluble protein (mg/g wet wt.)	IMP dehydrogenase	
		(nmol/h per mg of protein)	(% of activity of liver)
Thymus	47	24.3	1030
Spleen	62	20.0	850
Bone marrow	79	7.6	320
Testis	47	4.9	209
Liver	89	2.4	100
Brain	41	2.2	92
Lung	81	1.9	80
Kidney cortex	65	1.8	78
Peripheral leucocytes	96	1.4	61
Intestinal mucosa	57	0.7	29
Epididymal fat-pad	18	0.5	23
Skeletal muscle	43	0.04	2
Heart	46	0.03	1
Erythrocytes	148	0.01	<1

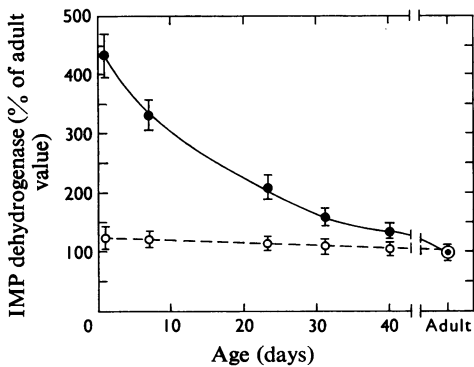


Fig. 5. *IMP dehydrogenase in livers of young rats* ●, Activity per unit wet weight (as percentage of adult value); ○, activity per cell (as percentage of adult value). Bars indicate  $\pm 1$  s.e. for five preparations.

protein; regenerating  $12.6 \pm 0.6 \mu\text{mol/h}$  per mg of protein. After 18h, activity slowly declined, as found in the earlier study (Jackson *et al.*, 1975). At no time did activity in the liver of sham-operated animals differ significantly from normal values of unoperated rats.

#### Studies with neonatal rats

A further system for the study of a non-malignant proliferating hepatocyte population is provided by the differentiating liver of newborn rats. These cells

have a doubling time of about 7 days (Weber *et al.*, 1971). Fig. 5 shows IMP dehydrogenase activity in the developing liver of the young rat. On the basis of activity per unit of wet weight, the neonatal value is more than four times that of the adult. However, the cell size of the neonatal hepatocytes is much less than that of the adult cells, so if the IMP dehydrogenase activity is expressed per mg of DNA, or activity per cell, the neonatal activity is 125% of the adult value, declining slowly with increasing age.

#### IMP dehydrogenase in malignant tissues

Earlier work showed increases of IMP dehydrogenase activity in 11 transplanted rat hepatomas. Measurements with three other hepatomas strengthened this conclusion. Specific activities in hepatomas 7787, 5123D and 7800 (transplant intervals 5.8, 1.8 and 1.2 months respectively) were 475, 680 and 569% of control liver values.

Activity of IMP dehydrogenase was also examined in the transplantable kidney tumours MK-1 and MK-3. Activity in these kidney tumours was increased 2.6–3-fold relative to normal kidney cortex. This suggests that the increase in IMP dehydrogenase may be a general feature of malignancy, and not restricted to liver neoplasia.

We decided to study whether the hepatoma IMP dehydrogenase had properties identical with those of the normal liver enzyme. Fig. 1 shows that the pH-activity profiles were similar. The 3924A hepatoma enzyme was eluted from DEAE-Sephadex, Sephadex G-150 and DE52 cellulose columns in the same

position as normal liver enzyme. Some kinetic properties of the hepatoma 3924A enzyme were as follows:  $K_a$  for IMP,  $12.5 \mu\text{M}$ ;  $K_b$  for  $\text{NAD}^+$ ,  $26.0 \mu\text{M}$ ;  $K_i$  for GMP,  $104 \mu\text{M}$ ,  $I_{0.5}$  for excess of  $\text{NAD}^+$ ,  $840 \mu\text{M}$ ;  $I_{0.5}$  for NADPH,  $178 \mu\text{M}$ . Although the liver

and hepatoma enzymes were not sufficiently pure for a comparison of molecular properties, these values, in conjunction with Table 2, show that, kinetically at least, the tumour enzyme did not have different properties from the liver enzyme.

#### Starvation and re-feeding

Earlier studies showed that during starvation an extensive breakdown of hepatic protein occurred; the activities of many hepatic enzymes declined under these conditions, but the enzymes of gluconeogenesis were selectively retained. The total number of cells in the liver does not change during starvation, so activities per cell give an indication of total hepatic activity (Weber *et al.*, 1972). The results of the present experiment are shown in Fig. 6. Total protein per liver cell decreased to 65% of control after 3 days of starvation. IMP dehydrogenase per cell, however, rose to 134% of control; thus the total liver content of this enzyme increased at a time when most hepatic protein concentrations were declining. Although this behaviour does not prove that IMP dehydrogenase is implicated in control of gluconeogenesis, to our knowledge such an increase in hepatic enzyme content during starvation has no precedent except for the enzymes of gluconeogenesis.

On re-feeding rats starved for 3 days, the total hepatic protein content rose to near the normal value after 2 days; the IMP dehydrogenase activity overshoot on day 1 of re-feeding, then fell to the control value in the subsequent 2 days. To examine the possibility that IMP dehydrogenase activity was selectively increased during starvation because of its postulated rate-limiting role for the biosynthesis of

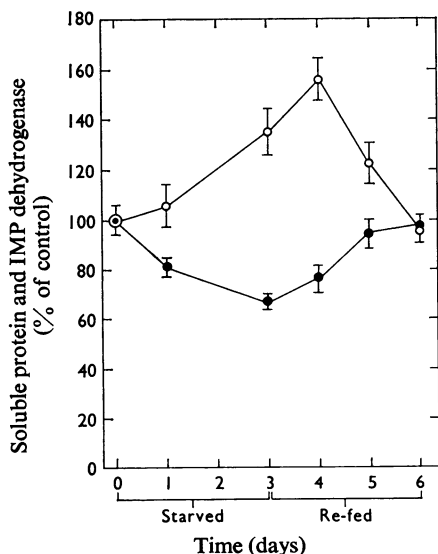


Fig. 6. IMP dehydrogenase in livers of starved and re-fed rats

Enzyme activity per cell (○) and total cytosol protein per cell (●) are plotted as percentage of normal values; bars indicate  $\pm$ S.E.M.

Table 4. Ribonucleotide contents of rat liver measured by ion-exchange chromatography

Nucleotide concentrations are means  $\pm$  S.E.M. of quadruplicate samples. Values in parentheses are a percentage of the appropriate control. \*Significantly different from control ( $P < 0.05$ ). Rats were lightly anaesthetized with diethyl ether, livers were freeze-clamped within 2s, 0.5 g of freeze-clamped powdered liver was extracted in 10 ml of 4.5%  $\text{HClO}_4$ , neutralized with KOH and 50  $\mu\text{l}$  samples were used for chromatographic nucleotide analysis, on a column (25 cm  $\times$  0.4 cm) of Partisil-SAX (Whatman, Clifton, NJ, U.S.A.). The column was eluted at 23°C with a flow rate of 2 ml/min with 0.38 M-potassium phosphate, pH 3.7, for 12 min, then with 0.42 M-potassium phosphate, pH 3.7. Retention times of standards were: UTP, 10.8 min; CTP, 13.0 min; ATP, 15.8 min; ITP, 21.0 min; GTP, 26.9 min.

	Nucleotide concentrations (nmol/g wet wt.)		
	Control	Starved 3 days	Starved 3 days then re-fed 1 day
ATP	2070 $\pm$ 60 (100%)	1740 $\pm$ 130 (71%)*	1820 $\pm$ 100 (88%)
ADP	860 $\pm$ 50 (100%)	860 $\pm$ 70 (100%)	810 $\pm$ 80 (95%)
GTP	277 $\pm$ 11 (100%)	321 $\pm$ 16 (116%)*	267 $\pm$ 9 (96%)
GDP	98 $\pm$ 7 (100%)	105 $\pm$ 6 (107%)	100 $\pm$ 2 (102%)
UTP	285 $\pm$ 30 (100%)	186 $\pm$ 9 (65%)*	287 $\pm$ 9 (101%)
UDP	108 $\pm$ 12 (100%)	71 $\pm$ 12 (66%)*	78 $\pm$ 5 (72%)*
CTP	52 $\pm$ 5 (100%)	53 $\pm$ 6 (102%)	54 $\pm$ 3 (104%)
CDP	40 $\pm$ 5 (100%)	37 $\pm$ 3 (92%)	38 $\pm$ 3 (95%)
ITP	<1	<1	<1
IDP	<1	<1	<1



GTP, which is required for gluconeogenesis, we measured ribonucleoside triphosphate concentrations in livers of starved and fed rats (Table 4). After 3 days of starvation, hepatic UTP and ATP concentration showed decreases, the CTP concentration was maintained unaltered, and that of GTP showed a small but significant increase. No detectable ITP was observed in liver from normal or starved rats and from the sensitivity of our method we may conclude that the ITP content of rat liver does not exceed 1 nmol/g wet wt. (Recoveries of added ITP and other triphosphates from the  $\text{HClO}_4$  extracts of freeze-clamped liver were between 93 and 100%.) On re-feeding, the hepatic nucleotides returned to normal value in 1 day.

### Discussion

Two kinds of IMP dehydrogenase have been described. The enzyme, in some micro-organisms, is an allosteric protein (Bussey & Levin, 1968; Ishii & Shiio, 1968; Nijkamp, 1969; Pourquie, 1969) but studies with mammalian enzymes, from mouse sarcoma 180 (Anderson & Sartorelli, 1968) and human placenta (Holmes *et al.*, 1974), showed hyperbolic kinetics. Our results indicated that the rat liver enzyme was of the latter type. Like both the sarcoma 180 and placenta enzymes, the rat liver IMP dehydrogenase showed an ordered, sequential kinetic mechanism with IMP the first substrate bound and XMP the last product released. Indeed, these three mammalian IMP dehydrogenases, although from different species, and from different tissues, show remarkable similarities (Table 2). It is possible that there is evolutionary pressure to conserve the properties of this enzyme. The enzyme from hepatoma 3924A appeared to resemble the normal liver enzyme in every respect examined, kinetic properties, pH optimum and susceptibility to inhibitors; there are thus no indications that the malignant transformation has altered the properties of the enzyme. The consistent increase in the IMP dehydrogenase activity of malignant tissues would confer on the tumours a greater capacity for GTP biosynthesis, possibly resulting in selective reproductive advantage for the cancer cells.

In a previous report of increased IMP dehydrogenase activity in a pathological condition, Pehlke *et al.* (1972) found a 5-fold increase in erythrocytes of patients with Lesch-Nyhan syndrome. Normal erythrocytes contained an inhibitor of IMP dehydrogenase lacking in the Lesch-Nyhan erythrocytes; dialysis of normal erythrocyte enzyme resulted in 4-fold activation, whereas enzyme from the Lesch-Nyhan patients showed little change. Our data exclude the possibility that a similar mechanism might account for the increase found in hepatomas. The increase in activity on dialysis was similar in extent for liver and hepatoma cells, and was due to removal of  $(\text{NH}_4)_2\text{SO}_4$ . More than 4h dialysis

resulted in loss of activity. Atkinson *et al.* (1963) noted that activity of IMP dehydrogenase from Ehrlich ascites cells, lost during dialysis, could be restored by re-addition of diffusate. In our liver studies, this was not the case. Further, if mixtures were made of liver and hepatoma enzyme preparations, the IMP dehydrogenase activity recovered was additive. We conclude that differences in activity between liver and hepatomas are the result of changes in the concentration of the enzyme, rather than changes in activities of activators or inhibitors.

The correlation between IMP dehydrogenase activity and cell proliferation, demonstrated in the hepatoma spectrum (Jackson *et al.*, 1975), was further emphasized by the results of the tissue-distribution study. In particular, the high activities found in thymus and spleen, and to a lesser extent in bone marrow and testis, indicated the importance of this enzyme for cell division. However, the lower activities in neonatal liver (relative to the per-cell activity of adult rat liver) and in intestinal epithelium, suggest that this relationship between IMP dehydrogenase activity and cell proliferation is not an obligatory one; perhaps these tissues derive a considerable portion of their GMP from the salvage pathway.

The increase of almost 6-fold in IMP dehydrogenase activity in regenerating liver again suggests the close link between this enzyme and cell division. It is notable how soon after the operation the activity started to rise; a significant increase was apparent at 6h, and the activity showed a peak at 18h. For rats of this size, the peak of DNA biosynthesis occurs between 20 and 24h after operation, and by this time the IMP dehydrogenase was already slowly declining. No further increase was apparent at the time of the second wave of DNA biosynthesis.

Several authors have commented on the very low absolute activity of IMP dehydrogenase in mammalian tissues (McFall & Magasanik, 1960; Atkinson *et al.*, 1964; Saccoccia & Miech, 1969). The present results are in line with their observations, and suggest that this enzyme may be one of the rate-limiting steps of nucleic acid biosynthesis. The activity in hepatoma 3924A, for example, is potentially sufficient to enable cellular RNA and DNA to be doubled in about 13h. Assuming an actual doubling time of 3 days, IMP dehydrogenase may operate physiologically at about 20% of its maximum velocity.

In liver of starved rats, where concentrations of all enzymes so far examined, except for those involved in gluconeogenesis, decline, the total IMP dehydrogenase content increased by 34%. Under these conditions hepatic concentrations of ATP and UTP showed decreases, CTP was unaltered and GTP showed a significant increase. These changes in IMP dehydrogenase and GTP concentration may be related to the requirement for GTP of the hepatic gluconeogenic enzyme phosphoenolpyruvate car-

boxykinase; succinyl-CoA synthetase, involved in conversion of 2-oxoglutarate into succinate, is also a GTP-requiring enzyme. Both these enzymes *in vitro* may utilize ITP as phosphate donor; however, the present work showed that no measurable amount of ITP was present in normal or starved rat liver, therefore the requirement for GTP is absolute.

We conclude that IMP dehydrogenase is one of the key rate-controlling enzymes of nucleic acid biosynthesis, that the activity shows a correlation with cell proliferation in both normal and malignant cell populations, and that the disproportionate increase found in malignant cells of rat liver and kidney may be linked with the neoplastic transformation.

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