The Distinctive Occurrence of Acid-Soluble Inosine in Rat Liver Microsomal Preparations. By Philip Siekevitz. (*From The Rockefeller Institute for Medical Research.*)*

It has recently been shown that freshly prepared rat liver mitochondria contain a distinct and constant complement of various acid-soluble nucleotides (1). Similar extracts of rat liver microsomes exhibit an entirely different picture, containing in measurable amounts only the nucleoside, inosine. In some recent studies young, Sprague-Dawley rats, obtained from the Holtzman Rat Co., Madison, Wisconsin, were decapitated, the livers excised, and approximately 12 gm. of wet weight immediately homogenized in cold 0.25 M sucrose solution. The nuclei, mitochondria, and debris were removed by centrifugation at 9000 *g* for 15 minutes in the Spinco centrifuge kept at 0°C. The supernatant was carefully removed, leaving behind the loosely packed layer lying on top of the mitochondria. The centrifugation of the supernatant at 105,000 *g* for 60 minutes gave a translucent reddish pellet which was then taken up in cold 0.25 M sucrose solution. Cold perchloric acid was then added to a final concentration of 0.5 M, and the precipitated protein suspension was left in the cold room for at least 2 hours. The protein was then sedimented, and to the supernatant solution was added cold 1.5 N KOH solution to the neutral point of phenol red. The resulting suspension of $KClO_4$ was left in the cold room overnight, then centrifuged, and the supernatant solution made slightly alkaline with a drop of NH_4OH. This solution was finally passed through a dowex 1 column, prepared in the formate form as previously described (1). Approximately 95 per cent of the material absorbing in the ultraviolet was held on the column. 5 ml. portions of the various eluants (*cf.* Fig. 1) were run through the column and the resulting typical picture is given in Fig. 1.

The only large peak, which is not found in the mitochondria from similar quantities of rat liver (1), was identified as inosine by the following methods. First, the tubes containing the compound were pooled and dried *in vacuo* at room temperature; the dried contents were taken up in water, made slightly alkaline, and placed on another dowex 1 formate column. The compound could be removed as a single peak with 0.02 M ammonium formate, pH 5. The ion exchange purification was repeated, this time removing the compound from the column as a single peak with 0.002 N formic acid. The pooled sample from this column was dried *in vacuo*, the dried contents taken up in water, and the following determinations were run on this solution. An ultraviolet absorption curve, with readings taken at every 2 mμ, showed a sharp peak at 250 mμ in acid (minimum at 220 to 226 mμ), with a shift to a broad peak at 256 to 260 mμ at alkaline pH (minimum at 230 mμ). The specific absorption was approximately 10 per cent higher at the alkaline maximum than at the acid maximum. The shapes of the curves agreed well with the data given by Hotchkiss (2) and by Beaven *et al.* (3) for an hypoxanthine-containing com-

* *Received for publication, June 2, 1955.*

pound. Spectral examination of the color obtained with the orcinol reaction (4) and of the color obtained with the cysteine-H_2SO_4 reaction (5) indicated the presence of a pentose sugar. The diphenylamine reaction (6) for desoxy sugars and the ninhydrin reaction (7) for amino acids were both negative. The test for inorganic phosphate (8) on an acid-digested sample was also negative. Using 13.1 × 10^{-3} as the molar absorption coefficient of inosine (3) and using a sample of xylose as a standard in the orcinol reaction, the following relation was obtained: 1.00 μM pentose/1.11 μM purine base/0.00 μM phosphate. Paper chromatography was performed by the method of Lofgren (9) using a butanol–isobutyric acid solvent.

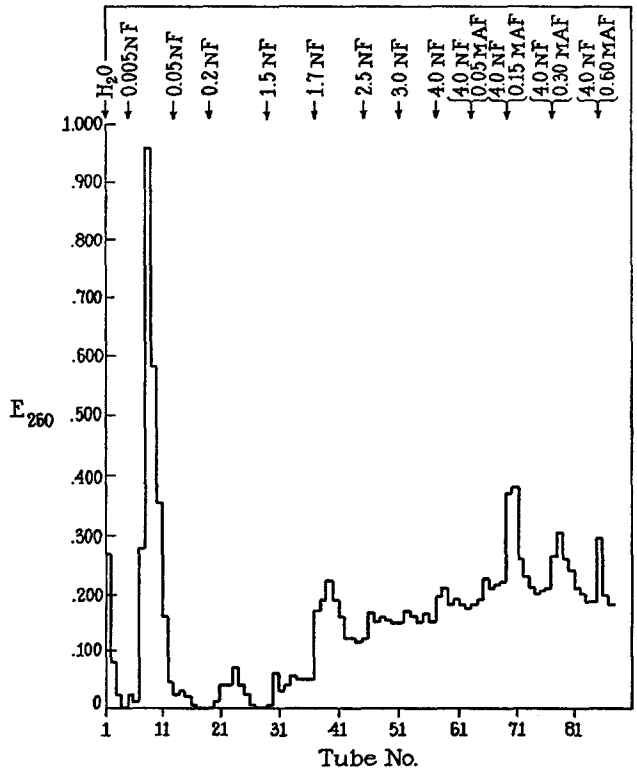

FIG. 1. Ion exchange chromatogram of acid-soluble compounds absorbing at 260 mμ obtained from rat liver microsomes. The preparation of the extract and of the column is given in the text. Each tube contained 5 ml. of the eluant given at the top of the figure. F = formic acid; AF = ammonium formate.

In this system, hypoxanthine, inosine, adenine, adenosine, xanthine, and xanthosine gave R_f values of 0.45, 0.26, 0.54, 0.70, 0.31, and 0.15 respectively. The compound from the microsomes gave a large ultraviolet-fluorescent spot with an R_f of 0.27 and a much smaller spot with an R_f of 0.43. Evidently some of the

inosine had broken down to hypoxanthine during the extraction and purification processes and this would account for the larger amount of purine base than of pentose sugar in the ratio given above. A known sample of inosine (Mann Research Laboratory, New York) came out at the same point on the dowex 1 column as the compound in the microsomes. Previous work (10) on similar chromatography of acid extracts of whole liver had failed to disclose inosine, and the reason probably was that at the neutral pH at which the extract was placed on the column (10), inosine, having a weakly dissociable H^+, would probably not have been retained.

Thus the large peak in Fig. 1 represents only inosine, and the determination of the area under this peak can be used as a quantitative estimation of the amount of inosine present. Calculations showed that there is 0.5 to 1.0 μM inosine in the microsomes from 10 gm. of wet weight of rat liver. This amount is similar to the amount of AMP, or ADP, or ATP found in the mitochondria from 10 gm. of wet weight of rat liver (1). As can be seen from Fig. 1 there are only much smaller amounts of other compounds absorbing in the ultraviolet, while the total amounts of those purine and pyrimidine bases and nucleosides which would not be held on the column or which can be washed off with water can be calculated to approximate only 20 per cent of the amount of inosine.

It is relevant to inquire about the nature of the inosine found in the microsomes. If the first microsomal sediment were homogenized in cold 0.25 M sucrose solution and immediately respun at 105,000 *g* for 60 minutes, approximately 10 per cent of the inosine (as determined by ion exchange chromatography) was lost from the pellet; if the suspension was diluted with the sucrose solution and kept at 0° for 30 minutes, about 35 per cent of the inosine was lost; while if this diluted suspension was incubated at 30° for 30 minutes, all of the inosine was lost from the pellet. Under the latter conditions of incubation 10 to 15 per cent of the protein, RNA, and phospholipide was lost from the microsomal pellet into the medium (11), and a similar treatment results in 50 to 100 per cent loss of the mitochondrial nucleotides (1). Thus the inosine is more readily lost than are the larger constitutents of the microsomes, but apparently less readily lost than if it were present as free inosine. In differential centrifugation experiments, the possibility always exists that some fractions are contaminated with components which are present in the cell in other fractions. To test the possibility that free inosine from the soluble portion of the cell contaminated the microsomes, 10 μM inosine was added to the supernatant (from 6 gm. of wet weight of liver) obtained after the mitochondria had been spun down. This supernatant was then centrifuged to obtain the microsomes and an extract of the microsomal pellet was made and tested for inosine by the ion exchange method. It was found that none of the added free inosine had centrifuged out with the microsomes. Also, the so called soluble portion of the homogenate, after the 60 minutes' microsomal centrifugation, was found in another experiment to contain about one-half as much inosine as did the microsomes from the same homogenate. It is therefore difficult to see how the inosine present in the microsomes could be a result of contamination from free inosine in the soluble part of the homogenate. It can be assumed, on the other hand, that the inosine in the soluble portion is due to the microsomal material still present there even after 60 minutes' centrifugation

(11). However, it is still possible that when the microsomes are separated by centrifugation, a soluble protein containing inosine is adsorbed onto the microsomal pellet, but this is unlikely because more than one-half of the protein-bound inosine would have to be adsorbed and this figure is far more than that found for any other protein (12). Also, under the same conditions of preparation, the microsomes contain no adenosine deaminase activity, this activity being found in the soluble part of the cell (*cf.* below). Thus it is probable that inosine does not exist as a freely diffusible compound in the microsomes, but that it is bound, though weakly, within the microsomal structure (11).

The possible routes of formation and the possible functions of microsomal inosine were also investigated. It has been found that there is a mechanism in the microsomes, but not in fresh mitochondria, which dephosphorylates IMP (and AMP)[1] to inosine (and adenosine). A previous finding (13) has been verified that adenosine deaminase occurs in the soluble portion of rat liver, and that AMP cannot be transformed by microsomes into inosine. No activity has been found for either 5′-adenylic acid deaminase or for xanthine oxidase systems in the microsomes. Though it has been stated (13) that nucleoside phosphorylase is located exclusively in the soluble portion of the cell, we have found some activity of the enzyme in both mitochondria and microsomes. However, both adenosine deaminase and nucleoside phosphorylase have been localized in the nuclei (14) as well as in the soluble part of the cell. Thus it would appear that the major pathway for the formation of inosine in the microsomes is by the dephosphorylation of IMP, and that the inosine formed there is not degraded to any great extent to hypoxanthine or to xanthine.

A recent report (15) indicated that inosine or hypoxanthine is involved in sulfite or cysteine oxidation in the liver. It was indeed found that cysteine oxidation, as measured manometrically, could be increased fourfold by the addition of inosine and not by the addition of adenosine, but this enzyme system is concentrated in the soluble portion of the cell. The presence of DPNH–cytochrome *c* reductase activity (16) and of an autooxidizable hemochromogen (17) (E'_0 of -0.12 volt) in the microsomes indicates the presence of an electron transport system there, and it is possible that the oxidation-reduction couple, inosine-xanthosine (E'_0 of -0.371 volt), plays a part in electron transport. However, the same microsomal preparation which could reduce cytochrome *c* in the presence of DPNH did not reduce it in the presence of DPN and inosine. The enzymatic function, if any, of microsomal inosine is therefore still unknown.

[1] IMP, inosinic acid; AMP, adenylic acid.

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