An enzyme with FAD pyrophosphatase activity was extracted from human placental syncytiotrophoblast microvilli and purified to near-homogeneity. The enzyme has been identified as 5'-nucleotidase by several criteria. Throughout purification, parallel increases in the specific activities of FAD pyrophosphatase and AMP phosphatase were observed. The enzyme was a glycoprotein with a subunit molecular weight of 74,000. EDTA treatment resulted in a marked decline in both activities, and restoration of FAD pyrophosphatase activity but not 5'-nucleotidase activity was accomplished by the addition of Co²⁺ or, to a lesser extent, Mn²⁺. The substrate specificity of the 5'-nucleotidase activity that we observed agreed closely with the results of others. The pyrophosphatase activity was relatively specific for FAD. ADP, ATP, NAD(H), and FMN were not hydrolyzed, and ADP strongly inhibited both activities. For FAD pyrophosphatase activity, a \( K_a \) of 1.2 × 10⁻⁶ M and a \( V_{max} \) of 1.1 \( \mu \)mol/min/mg protein were determined in assays performed in the presence of Co²⁺. In the absence of added Co²⁺, the \( V_{max} \) declined but the \( K_a \) was unchanged. For 5'-nucleotidase (AMP as substrate) the \( K_a \) was 4.1 × 10⁻⁸ M and the \( V_{max} \) 109 \( \mu \)mol/min/mg protein. Hydrolysis of FMN to riboflavin was observed in partially purified detergent extracts of microvilli that contained alkaline phosphatase activity and lacked FAD pyrophosphatase and 5'-nucleotidase activity. The presence of both FAD pyrophosphatase and FMN phosphatase activities in syncytiotrophoblast microvilli supports the view that the placental uptake of vitamin B₂ involves the hydrolysis of FAD and FMN to riboflavin which is then absorbed, a sequence postulated for intestinal absorption and liver uptake.

5'-Nucleotidase (EC 3.1.3.5, 5'-ribonucleotide phosphohydrolase) is an intrinsic protein present as an ectoenzyme in the plasma membrane of most nucleated mammalian cells (1-3). The activity has also been found in the cytoplasm (4) and in lysosomes (5). The function of the enzyme is unknown, but may involve the cellular uptake of nucleoside derivatives of a variety of nucleoside 5'-monophosphates (6, 7). 5'-Nucleotidase, presumably the plasma membrane enzyme, has been purified from homogenates of human placenta and some of its properties investigated (8-12). A subunit molecular weight of 73,000-78,000 has been observed on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10, 12). The activity of the enzyme is inhibited by lectins, which bind to the enzyme (9, 11), and by EDTA (11).

In preliminary studies of the uptake of vitamin B₂ by the human placenta, we observed that substantial quantities of radioactively labeled FMN and riboflavin were produced when [³H] FAD was incubated with a fraction rich in microvilli extracted from the syncytiotrophoblast surface. This surface, which forms the maternal-fetal interface, is in direct contact with maternal blood. Studies were then undertaken to identify the enzyme responsible for the hydrolysis of FAD, and the results of these studies, which indicated that plasma membrane 5'-nucleotidase of human placenta possesses FAD pyrophosphatase activity, form the basis of this report.

**EXPERIMENTAL PROCEDURES AND RESULTS**

**Subcellular Distribution of FAD Pyrophosphatase Activity**

The specific activity of FAD pyrophosphatase in a homogenate of placenta and in various subcellular fractions is given in Table I. The microsomal fraction of the homogenate displayed a much higher specific activity than the homogenate or the mitochondrial and soluble fractions. Detached syncytiotrophoblast microvilli are sedimented at 105,000 × g and would be present in the microsomal fraction. The microvillus fraction, obtained from a portion of the placenta not used for homogenization, showed the highest specific activity. The subcellular distribution of FAD pyrophosphatase activity closely paralleled that of 5'-nucleotidase as reported by Truman et al. (15).

**Inhibitors**—Sodium phosphate (0.1 mM), sodium pyrophosphate (5 \( \mu \)M), and NAD⁺ (5 \( \mu \)M) were not inhibitory to FAD pyrophosphatase activity. The inhibitory effects of AMP, ADP, and ATP on FAD pyrophosphatase activity are shown in Fig. 4. The inhibition by ADP was essentially complete at a concentration of 5 \( \mu \)M. The apparent inhibitory effect of ATP may have been due, in part, to ADP produced by spontaneous hydrolysis during the incubation period.

**Heat Stability**—A gradual decrease in FAD pyrophosphatase activity was observed when the enzyme, partially purified by ion-exchange chromatography (Fig. 1), was maintained at 56 °C (Fig. 5). In contrast, no decline in alkaline phosphatase and FMN phosphatase activities was observed after 30 min at 56 °C when the pooled fraction from the ion-exchange column that contained these activities was tested. Similar

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† Recipient of a Postgraduate Scholarship from the Medical Research Council of New Zealand.

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pared according to the method described by Truman et al. Different portions of the same placenta were used for homogenization and to obtain microvilli. FAD pyrophosphatase activity was assayed as described under "Experimental Procedures" except that Co\(^{2+}\) was not included in the assay mix.

The placental homogenate and the subcellular fractions were prepared according to the method described by Truman et al. (15).

**TABLE I**

| Fraction    | FAD pyrophosphatase activity (pmol/min/mg protein) |
|-------------|-----------------------------------------------------|
| Homogenate  | 69                                                  |
| Mitochondrial | 162                                               |
| Microsomal  | 487                                                 |
| Soluble     | 184                                                 |
| Microvilli  | 714                                                 |

**Discussion**

5'-Nucleotidase has been extracted from human placental syncytiotrophoblast microvilli and purified to near-homogeneity. Several properties which we observed agree with the reports of others for the placental enzyme. The subunit molecular weight of 74,000 that we observed is close to the 75,000 reported by Thompson et al. (12), and the substrate specificities that we found are very similar to those described by two other groups (Table V). The enzyme is known to be EDTA-sensitive, and Maguire et al. (11) and Dornand et al. (37), like us, were unable fully to restore activity by the addition of divalent cations. The enzyme is a glycoprotein that binds concanavalin A (9, 11).

The only other report that we are aware of concerning the amino acid composition of a membrane-bound eukaryotic 5'-nucleotidase is that of Dieckhoff et al. (38) for an enzyme purified from chicken gizzard smooth muscle. An unusual composition was reported with serine, glycine, and glutamic acid/glutamate making up well over half the total amino acids; threonine and cysteine were apparently absent. The amino acid composition of the human placental enzyme is more usual (Table VI), and the finding that over one-third of the amino acids are hydrophobic is consistent with its location in the plasma membrane. The small number of half-cystine residues also suggests that few intra- or intermolecular disulfide bonds exist in the protein molecule.

A remarkable property of the placental 5'-nucleotidase was its FAD pyrophosphatase activity which was stimulated by Co\(^{2+}\) and, to a lesser extent, by Mn\(^{2+}\) under conditions where AMP hydrolysis was either inhibited (with Co\(^{2+}\)) or unaffected (with Mn\(^{2+}\)). Stimulation of the FAD pyrophosphatase activity by Co\(^{2+}\) was observed at all steps of the purification procedure, from the homogenate of placenta to the final preparation after affinity chromatography. It is, therefore, unlikely that the increase in activity in the presence of added Co\(^{2+}\) was due to the replacement of divalent metal ions lost during the purification procedure. In contrast to several yeast and bacterial enzymes which possess wide-ranging phosphatase and pyrophosphatase activities (1), the reaction with FAD seemed relatively specific; FMN, NAD(H), ADP, and ATP were not substrates. It is of interest that Fox and Marchant (8) reported the hydrolysis of UDP-glucose by a 5'-nucleotidase present in a microsomal fraction from human placenta. Co\(^{2+}\) protected the enzyme from heat inactivation.

5'-Nucleotidase activity displayed a broad pH optimum from pH 6.5 to 8.5. There was no apparent difference in the pH optimum, whether the assays were performed in the absence or presence of 25 mM Co\(^{2+}\). FAD nucleotidase activity had a similarly broad pH optimum from pH 6 to 8.5 when AMP was used as substrate. However, we did not observe a second pH optimum between pH 9 and 10 when the assays were performed in the presence of 1 mM Mg\(^{2+}\), as reported by others (11, 36).

The only other property that we are aware of concerning the amino acid composition of a membrane-bound eukaryotic 5'-nucleotidase is that of Dieckhoff et al. (38) for an enzyme purified from chicken gizzard smooth muscle. An unusual composition was reported with serine, glycine, and glutamic acid/glutamate making up well over half the total amino acids; threonine and cysteine were apparently absent. The amino acid composition of the human placental enzyme is more usual (Table VI), and the finding that over one-third of the amino acids are hydrophobic is consistent with its location in the plasma membrane. The small number of half-cystine residues also suggests that few intra- or intermolecular disulfide bonds exist in the protein molecule.

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in the absence of substrate, which suggested that the cation could interact with the enzyme in the absence of FAD. However, the mechanism of the stimulation of FAD pyrophosphatase activity by Co²⁺ and Mn²⁺ was not specifically investigated.

Some other properties of the FAD pyrophosphatase activity deserve brief comment. The strong inhibitory effect of ADP contrasted with the lack of inhibition by FMN, which represents one-half of the FAD molecule and is a product of the FAD pyrophosphatase reaction. Lumiflavin, the isocolaloxazine portion of the FAD molecule, also had no effect on the FAD pyrophosphatase activity (results not shown). AMP, the other half of the FAD molecule, a product of the pyrophosphatase reaction and a substrate of the 5'-nucleotidase activity of the enzyme, was far less inhibitory for the pyrophosphatase activity than was ADP. It is possible that part of the FAD molecule represented by ADP is important in the binding of FAD to the enzyme. These observations may also explain the failure of the enzyme to bind to ADP-agarose, in which ADP was attached to the beaded agarose through the ribose hydroxyls (results not shown).

The mechanisms of uptake and transport of flavin vitamers by the human placenta have not been clearly delineated. A pregnancy-specific, riboflavin-specific binding protein in human and rat maternal serum has been described (39, 40) which, in the rat, appears to play a vital role in the placental transfer of riboflavin. Normal human immunoglobulins, probably immunoglobulin G, bind riboflavin and FAD with very high affinity (41), and it may be that flavin vitamers cross the placenta bound to immunoglobulin G, as has been observed for insulin (42). From the results of in vitro perfusion of a human placental lobule, Dancis et al. (43) concluded that riboflavin was actively transported from maternal blood to the fetus; studies of the transport of FAD and FMN were not reported. Our observation that 5'-nucleotidase of syncytiotrophoblast microvilli possesses FAD pyrophosphatase activity together with previous reports that FMN is a substrate for alkaline phosphatase (44) and our own observation that microvilli of the term placenta contain abundant FMN phosphatase activity make it unlikely that free FAD or free FMN are taken up from maternal blood by the placenta and transported to the fetus unchanged. Our findings favor the view that free FAD and free FMN are hydrolyzed to riboflavin which is then absorbed—a mechanism postulated for intestinal absorption (17, 45, 46) and liver uptake (47). Whether or not flavins bound to protein in maternal blood are used by the placenta or are transported to the human fetus are unanswered questions.

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FAD Pyrophosphatase Activity of 5'-Nucleotidase

Supplementary Material 5

Supplementary Table 1: Purification of FAD pyrophosphatase from tobacco leaves.

The procedures used were described in "Experimental Procedures".

Table 1: Purification of FAD pyrophosphatase from tobacco leaves.

| Step | Protein | Specific Activity | Yield (mg) |
|------|---------|------------------|------------|
| 1    | 40,000  | 0.25             | 0.04       |
| 2    | 40,000  | 0.5              | 0.12       |
| 3    | 40,000  | 2.4              | 0.04       |
| 4    | 40,000  | 10               | 0.12       |

Note: The specific activity and yield are in mg of enzyme activity per mg of protein.

Additional Information:

1. The procedures used were described in "Experimental Procedures".
2. The procedures used were described in "Experimental Procedures".
3. The procedures used were described in "Experimental Procedures".
4. The procedures used were described in "Experimental Procedures".
5. The procedures used were described in "Experimental Procedures".

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### Table III

| Treatment | Activity relative to untreated sample (x) |
|-----------|------------------------------------------|
| FAD alone | 22                                        |
| FAD alone: Ca²⁺ | 22                                        |
| FAD alone: Mg²⁺ | 22                                        |
| FAD alone: Ca²⁺ | 22                                        |
| FAD alone: Mg²⁺ | 22                                        |
| FAD alone: Ca²⁺ | 22                                        |
| FAD alone: Mg²⁺ | 22                                        |

### Table IV

| Treatment | % Activity of control |
|-----------|-----------------------|
| Untreated | 46                     |
| FAD alone | 22                     |
| FAD alone: Ca²⁺ | 157                   |
| FAD alone: Mg²⁺ | 307                   |

| Substrate | Percentage of FAD: Pyrophosphatase activity (nmol 5'-Nucleotide activity) |
|-----------|--------------------------------------------------------------------------|
| AMP       | 46                                                                       |
| ADP       | 37                                                                       |
| ATP       | 32                                                                       |
| GTP       | 22                                                                       |
| TTP       | 7                                                                        |
| CTP       | 4                                                                        |

### Table V

### Figure 1

[Image of a chromatogram, possibly showing the separation of nucleotides or related compounds.]
FAD Pyrophosphatase Activity of 5'-Nucleotidase

FIG. 2. Affinity chromatography on G-agarose. Experimental details are given in the text. 
- - , 5'-nucleotidase activity; --- , FAD pyrophosphatase activity; 
NaCl concentration.

FIG. 3. Results of electrophoresis. Experimental details are given in the text. A, SDS-polyacrylamide gel electrophoresis during enzyme purification. 1, placental microsomal preparation; 2, detergent extract of microsomal membrane proteins. Samples for gels 3-5 were taken from pooled fractions that contained FAD pyrophosphatase and 5'-nucleotidase activity. 3, after column chromatography on DEAE-Sephadex A-50; 4, after column chromatography on DEAE-Sephadex A-50 and 5, after affinity chromatography on FAD-agarose. Gels 1 and 2 were stained with Coomassie blue. B, results of SDS-polyacrylamide gel electrophoresis of a sample from a pooled fraction of FAD pyrophosphatase/ 
5'-nucleotidase after chromatography on FAD-agarose. The gel was silver-stained.