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Short Communications

Flavin adenine dinucleotide (FAD) metabolism and lactation

The form in which riboflavin occurs in milk has been shown to vary with species. DAVIS *et al.*[1] suggested that while riboflavin occurs free in cow milk, at least part of that occurring in sow milk is in a combined form, from which free riboflavin is readily liberated. This has recently been confirmed by MODI AND OWEN[2], who also showed that the riboflavin in sow milk is almost exclusively in the combined form, identified by them as FAD. In cow milk, however, free riboflavin was shown to be accompanied by only relatively small amounts of FAD. Whether the FAD is itself combined or free, was not investigated. Combined FAD is an active component of a number of enzyme systems, and hence species differences in its distribution may in turn reflect wider metabolic differences. Studies have therefore been made of the occurrence and metabolism of FAD in the lactating sow and cow.

Free FAD in milk and in blood was determined by the manometric method of OCHOA AND ROSSITER[3]. The sample of milk or blood to be determined, or a standard solution of FAD, was diluted to 2 ml with $M/15$ sodium pyrophosphate buffer, pH 8.3, and placed in the main chamber of a Warburg vessel along with 1 ml of a solution in the same buffer of the specific protein of D-amino acid oxidase[4]. The central well of the vessel contained filter paper moistened with 0.2 ml N alkali, and the side limb 0.2 ml of 4.5% DL-alanine in pyrophosphate buffer. The alanine was then mixed with the contents of the main chamber, the flask shaken at 37° C for 15 min, and the rate of oxygen uptake measured during the following 30 min. Under these conditions, 0.5 μg FAD produced an oxygen uptake of approximately 80 μl, the rate being constant over the 30 min period. The relationship between oxygen uptake and FAD present was linear for quantities up to 1.0 μg.

Milk from individual cows and from individual sows after injection with oxytocin was ultrafiltered[5], and the FAD present in 2 ml portions of the filtrate determined. Free FAD was absent from all samples of ultrafiltrate from cow milk, while sow milk contained 0.15–0.20 μg/ml. This was confirmed qualitatively by paper chromatographic examination. When chromatographed on Whatman No. 31 extra thick paper using the butanol/acetic acid/water system of CRAMMER[6], concentrated ultrafiltrate from cow milk, in all cases, yielded only fluorescent spots corresponding to riboflavin and riboflavin-5′-phosphate. With similar material from sow milk, however, a spot corresponding to FAD was obtained. In most cases this was unaccompanied by riboflavin or

riboflavin-5′-phosphate, but occasionally traces of these substances, arising presumably during concentration or chromatography, were detected.

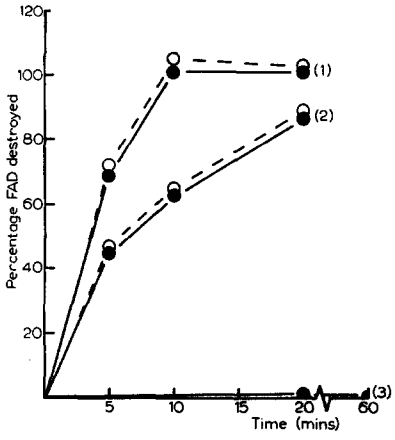

Fig. 1. Rate of destruction of FAD added to (1) cow milk, (2) pasteurised cow milk, and (3) sow milk. The values obtained by direct determination of residual FAD are indicated by ●—●, and those by calculation from determination of riboflavin produced, by O—O.

When fresh samples of sow and cow milk were heated for 3 min at 95° C and the precipitated protein in the sow milk samples removed by centrifugation, free FAD was found to be present. The concentrations were approximately 0.8 μg/ml in sow milk and 0.3 μg/ml in bovine milk. Thus there is present in the milk of each species a form of FAD which is not utilised by the specific protein of D-amino acid oxidase, but which is readily converted to a usable form by heating. FAD can therefore be considered to be bound to one or more of the milk proteins which on heating become denatured and liberate free FAD. Furthermore this protein-bound form accounts for all the FAD of cow milk but for only part of that in sow milk, the remainder being in the free state.

The absence of free FAD from cow milk was studied by investigating the fate of FAD added to raw milk from both species. 2 ml samples of cow and sow milk were each incubated at 37° C with 20 μg FAD for 20 min and 60 min respectively. After selected intervals during these periods, the amounts of FAD present in trichloroacetic acid extracts of the samples were determined by the method of BURCH, BESSEY AND LOWRY[7]. In a parallel set of samples, the combined riboflavin and riboflavin-5′-phosphate contents, expressed as riboflavin, were determined after the same time intervals, by the fluorimetric method of the same authors[7]. A typical set of results is shown in Fig. 1. From this it is clear that, although sow milk is apparently without effect on FAD, cow milk causes its rapid decomposition. Furthermore, at any given time the amount of FAD destroyed is equivalent to the combined amounts of riboflavin and its phosphate produced. Paper chromatographic examination of those cow milk samples incubated for 20 min showed that only traces of riboflavin phosphate were present, and that the main product of the decomposition of FAD was riboflavin.

When the experiment was repeated using pasteurised cow milk samples which showed no phosphatase activity[8], the rate of disappearance of added FAD, shown in Fig. 1, was considerably less. While this disappearance was again exactly balanced by production of riboflavin and its phosphate, paper chromatography now showed riboflavin phosphate to be the main decomposition product. It can therefore be concluded that there exists in cow milk a system of enzymes capable of converting FAD to riboflavin, in a number of steps the last of which is the dephosphorylation of riboflavin-5′-phosphate, and that no such system exists in sow milk. The possibility that the stability of FAD added to sow milk was due to its adsorption by protein thus preventing enzymic attack can be discarded, since free FAD has already been shown to be a constituent of sow milk. During normal conditions of lactation, therefore, free FAD arising in cow milk could be expected to be rapidly converted to riboflavin, whereas in sow milk it would remain unaffected.

In bovine milk, at least part of the combined form of FAD is present as xanthine oxidase[9]. However, no xanthine oxidase activity could be demonstrated in sow milk using the method of ZITTLE *et al.*[10], and when the inactive sow milk was mixed with cow milk, the xanthine oxidase activity of the cow milk was unaffected. The lack of enzyme activity of sow milk was therefore not due to the presence of an inhibitor but to the absence of xanthine oxidase, and hence the combined FAD of sow milk is not identical with that of cow milk.

A preliminary study has been made of the FAD in the blood of the sow and the cow to determine whether blood variations could account for those species differences mentioned above. Heparinised blood from the jugular vein was used, and the FAD was determined enzymically, as already described. It was found that if standard amounts of FAD were incubated with blood and the specific protein of D-amino acid oxidase, wide variations in oxygen uptake were obtained depending upon the order in which the reactants were added to the incubation vessel. When FAD and enzyme solutions were mixed before addition of the blood, concordant values for the rate of oxygen consumption were always obtained. From this it appeared likely that the blood of both species was able to convert added FAD to a substance which could not be utilised by the specific protein of D-amino acid oxidase. This was confirmed in the following way. 2 ml samples of cow and sow blood were first incubated with 0.5 μg FAD in 0.1 ml pyrophosphate buffer at 37° C for 0.5 and 1.5 h. Immediately after this incubation, the free FAD present was determined in the usual way. The

results are listed in Table I along with those obtained when the determination was carried out without this preliminary incubation.

TABLE I

DECOMPOSITION OF FAD IN COW AND SOW BLOOD

Period of incubation of blood and FAD (h)	*O_2 uptake in µl/0.5 h*			
	Sow blood		*Cow blood*	
0.0	76.0	78.5	82.5	79.0
0.5	4.5	5.8	3.9	3.2
1.5	—	—	1.5	2.1

From the figures listed in Table I it is apparent that *in vitro*, uncombined FAD is rapidly destroyed both in cow and sow blood. The complete absence of free FAD cannot however be assumed, for although no free FAD was detected by enzymic assay of 2 ml samples of blood from both species, the method, under the present conditions, could be expected to detect only amounts greater than 0.02 µg/ml. The presence of FAD at concentrations considerably less than this could easily account for the amount found free in sow milk, and for a substantial part of the riboflavin of cow milk. Although free FAD was not detected in fresh blood, samples assayed after being heated at 95° C for 3 min yielded uptakes of oxygen equivalent to FAD concentrations of 0.43 and 0.63 µg/ml in cow and sow blood respectively. These figures presumably represent the concentrations of FAD combined with protein, and are in agreement with the values reported for ox blood by OCHOA AND ROSSITER[3]. No attempt has yet been made to characterise further the combined FAD from either source although it appears unlikely that either contains xanthine oxidase activity. This being so, the xanthine oxidase of cow milk would appear to arise by elaboration of the blood flavoproteins within the mammary gland during lactation, while sow blood, containing a similar concentration of flavoproteins, is not similarly converted.

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Sur les interactions de la lipase pancréatique avec les triglycérides

D'après WILLSTAETTER ET WALDSCHMIDT-LEITZ[1], la lipase pancréatique en solution aqueuse s'adsorbe partiellement sur une fine dispersion de tristéarine et se laisse ensuite éluer par le phosphate d'ammonium. Ce phénomène est intéressant car il peut servir à purifier la lipase et à mieux comprendre ses interactions spécifiques avec des substrats insolubles dans l'eau.

Des essais d'adsorption ont été effectués avec une préparation purifiée de lipase (préparation C_2* de notre précédente publication[2]). 300 mg de tristéarine en poudre ont été agités chaquefois

* Cette préparation est obtenue en précipitant 2 fois par Am_2SO_4 un extrait aqueux de pancréatine de porc.