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DIMINUTION IN STARVATION OF CAPACITY OF CELL SAP TO SUPPORT PROTEIN SYNTHESIS IN CELL-FREE SYSTEMS

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1. Introduction

A diminished capacity of sap from tissues of diabetic animals to support incorporation of amino acids into protein in cell-free systems has several times been noted [1–3]. Conversely there is evidence that long-term protein malnutrition enhances the activity of the hepatic amino acid activating enzymes [4, 5]. There are surprisingly no reports of the effect of fasting on the capacity of sap to support protein synthesis by isolated ribosomes, though after 6 days without food the hepatic content of amino acid activating enzymes is said to be unchanged [4].

We find that sap from liver and muscle of fasted rats has a lower capacity to support protein synthesis and that this decrease is not attributable to change in the size of the pool of free amino acids.

2. Methods

Sap and ribosomes were prepared from liver of rats of about 120 g wt that had either free access to food or had food withdrawn 48 hr previously. Diabetes was induced by intravenous injection of alloxan (65 mg/kg) 60 hr previously.

Livers were homogenised at 4° with a Dounce homogeniser in 2.5 vol medium containing 50 mM tris-HCl, pH 7.6, 100 mM KCl, 5 mM MgCl, 0.25 M sucrose and 6 mM mercaptoethanol. The homogenate was centrifuged at 10,000 *g* for 15 min and the supernatant treated with α-amylase (Worthington Biochemical Corp. – 50 units per ml supernatant [6]) to degrade any glycogen present. The portion of the supernatant that was to become the sap was then centrifuged at 150,000 *g* for 1 hr and the supernatant collected, care being taken to avoid gathering any lipid at the top of the tube. The portion of the 100,000 g supernatant that was to be used as a source of ribosomes was made 1% with respect to deoxycholate and then layered over buffer containing 1 M sucrose and also centrifuged for 1 hr at 150,000 *g*. The ribosomal pellet was suspended in 2 ml of buffer, centrifuged lightly and aliquots of supernatant taken for determination of RNA [7].

The cell sap was either used directly or passed through a column of Sephadex G25 equilibrated with buffer [8], during which some dilution took place. For preparation of 'pH 5 fraction' 1 N acetic acid was added slowly with stirring to 6–8 ml of sap until the pH fell to 5. The precipitate was collected, washed once by suspension in 0.25 M sucrose in water [9], then redissolved in buffer equal in volume to 1/5 of that of supernatant used for precipitation. Protein

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Table 1
Effect of various factors on incorporation of leucine into protein by liver ribosomes.

System	Ribosome suspension (ml)	Incorporation into protein (cpm)	(%)
Complete	0.10	4358	100
	0.05	2170	53
	0.02	875	19
	0.01	440	11
	0	150	5
No sap	0.10	141	4
No ATP and GTP	0.10	3519	82
No PEP	0.10	2842	61

The complete system is as described in the methods section and contained 0.1 ml sap. In the experiment indicated the ribosomal suspension contained 2.4 mg RNA/ml. The percentage changes are the average of 5 separate experiments.

Table 2
Capacity of ribosomes and sap from fed and fasted rats to incorporate leucine into protein.

Sap from liver of rats fasted (days)	Ribosomes prepared from animals fasted (days) 0	1	2
0	686	655	524
1	577	506	460
2	559	513	472
Sap from muscle of rats fasted (days)			
0	597	553	521
1	374	365	310
2	433	372	357

Incorporation is expressed as pmole leucine incorporated per mg ribosomal RNA. 0.1 ml of sap was added to each tube.

content of sap and pH 5 fraction was measured by the method of Lowry et al. [10].

The incubation medium for incorporation of leucine into protein contained in addition to the other constituents of the homogenising medium: 1 mM ATP, 0.1 mM GTP, 5 mM phosphoenolpyruvate, additional $MgCl_2$ to 6 mM (free Mg^{2+} likely to be around 3.2 mM [11]), as well as various amounts of sap and ribosomal suspension, and 1-^{14}C-leucine added at a concentration of either 15 or 100 μM (about 0.4 μCi per tube), total vol. 0.5 ml. Incubation was for 30 min at 37°, after which 2 ml of 10% TCA containing 1 mg/ml DL-leucine was added. The precipitate was collected and resuspended in 3 ml of the TCA/leucine solution, heated for 15 min at 90° then collected on glass fibre discs (Whatman) and further washed with TCA. The discs were placed in 10 ml scintillation fluid (toluene–Triton X-100, 2:1, containing 0.4% BBOT), which dislodged the protein from the discs, and the radioactivity counted at about 70% efficiency.

3. Results.

In initial experiments the incorporating system contained 0.1 ml of sap (equivalent to about 30 mg of liver). The amount of leucine incorporated was proportional to the quantity of ribosomes added in the range up to about 250 μg per tube of ribosomal RNA (table 1). Incorporation was substantially less when phosphoenolpyruvate was omitted, but was not seriously affected by omission of ATP and GTP, presumably because the sap contained appreciable nucleotide only in need of a regenerating system. No pyruvate kinase was added since the sap contains adequate endogenous activity (unpublished observations). Omission of ribosomes or of a sap reduced incorporation to near zero levels.

Table 2 shows the results of an experiment in which the activity of sap prepared from both liver and muscle of animals fasted for various periods is studied in conjunction with hepatic ribosomes, the ribosomes also being used in the similar conditions. There is consistent diminution in the activity of sap from both tissues from fasting animals to support incorporation as well as decrease in the activity of the ribosomes. The diminished activity of ribosomes from liver of fasted rats has been previously observed [12, 13], but the change in activity of the sap appears not to have been noted and merited further attention. The difference if even greater when the figures are correc-

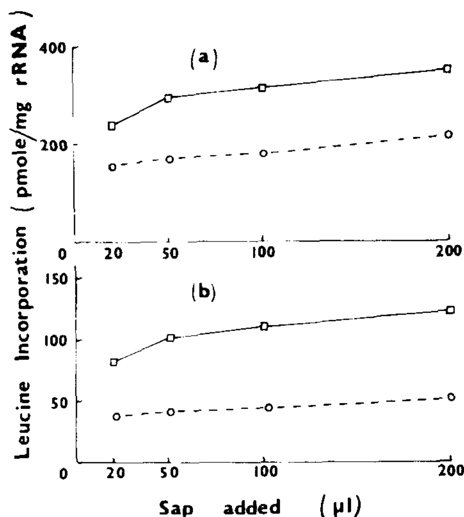

Fig. 1. Effect of various amounts of sap from liver of fed and fasted rats on the incorporation of leucine into protein by hepatic ribosomes. □—□ sap from fed rats; ○---○ fasted for 48 hr. In (a) incorporation is not corrected for the different amounts of protein in the two saps; in (b) the results of (a) are divided by the quantity of protein (mg) in 0.1 ml of the appropriate sap.

ted to comparable amounts of protein as in the experiment in fig. 1. In fact sap from fasted animals normally contained more protein than that from fed sources, presumably because of the lower glycogen in the tissue. Variation in the amount of sap added did not make a large difference in the amount of incorporation (fig. 1). Saturation of the system with respect to sap could be a correct interpretation of these findings (though less than the critical 25:1 ratio of sap:ribosomal protein noted by Munro et al. [8] was used), but similar results could arise if leucine added with the sap diluted the specific activity of the labelled leucine. 30 mg of tissue contains roughly 8 nmoles of free leucine, which is about the same amount as that added in labelled form. Diminution in incorporation with the fasted sap could arise if the dilution were greater (though there is little evidence for enhanced leucine levels in fasting liver [14–16]). In order to clarify the point the experiments were repeated using sap which had been passed through Sephadex to remove low molecular weight contaminants and by increasing the amount of added leucine to the point where dilution was not likely to pose a serious problem.

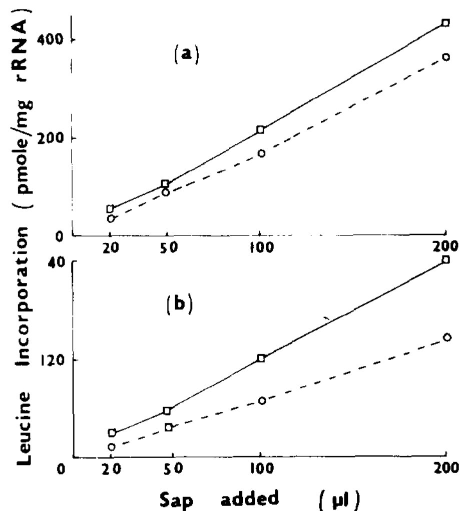

Fig. 2. Effect of sap after Sephadex filtration on its ability to support incorporation of leucine by hepatic ribosomes. Details as in fig. 1

Fig. 2a shows that after passage of the sap through Sephadex incorporation is proportional to the amount of sap added and that the difference between the sap from fed and fasted rats persists. (Again, the difference is greater then the values are corrected to comparable amounts of protein (fig. 2b). Moreover similar diffe-

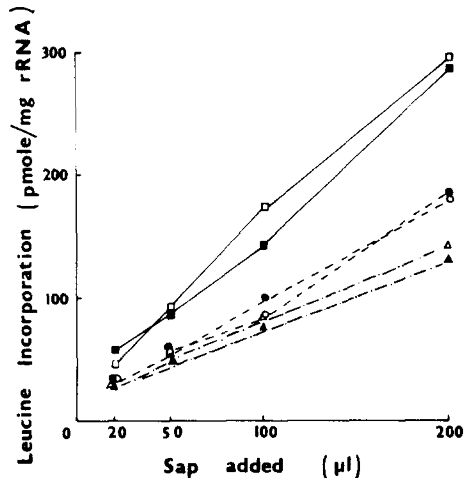

Fig. 3. Effects of sap from fed, fasted and diabetic liver, both before (open symbols) and after (closed symbols) Sephadex filtration, to support incorporation by hepatic ribosomes of leucine added at saturating concentration (0.1 mM). □—□, sap from fed; ○---○, fasted; △—△, diabetic liver. Note that incorporation is corrected for different amounts of protein in the sap.

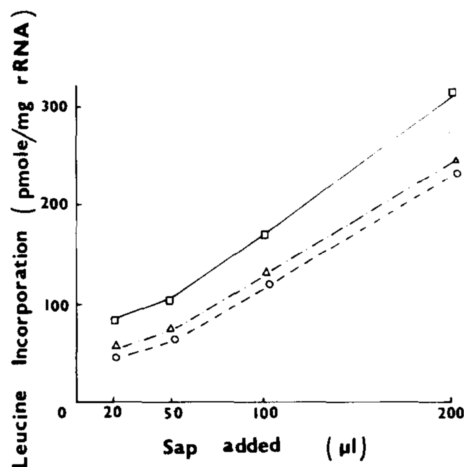

Fig. 4. Effects of 'pH 5 enzyme' fraction, prepared from sap of fed, fasted and diabetic liver to support incorporation by hepatic ribosomes. □——□ sap from fed; ○- - -○, fasted and △- - -△, diabetic livers. The amount of incorporation has been divided by the quantity of protein (mg) in 0.1 ml of the appropriate pH 5 fraction. Pyruvate kinase (Boehringer 50 µg) was added to each tube.

rences were found when the concentration of leucine was raised (fig. 3). Under these conditions the difference between the capacity of untreated sap and sap passed through Sephadex declines, which suggests that Sephadex treatment primarily diminishes dilution of the specific activity of the added amino acid as opposed to removing putative inhibitors present in the sap [8]. This last experiment included sap from severely ketotic alloxan diabetic rats which possessed a lower activity by comparison with the fed normal and also with the fasted sap.

The differences between sap from fed and fasted rats persists when the pH fraction of the sap is used in place of whole sap (fig. 4). It should be noted here that the diminished capacity is seen only when expressed in terms of unit quantity of pH 5 fraction; fasted sap usually produced a larger quantity of protein which in total exhibited greater support for incorporation. There was also less specific activity in the precipitate from diabetic sap compared with fed sap.

4. Discussion

The present results confirm earlier findings [8] that the in vitro system is not saturated with sap at ratios of sap to ribosome protein of around 10:1 such as have been used here, though this may not be apparent unless sap is treated with Sephadex. That the system should not be saturated is presumably necessary in order for a difference in functional capacity to be detectable and the use of differing ratios of sap or pH 5 proteins to ribosomes may explain some of the variability in extent of change of function in diabetes [1–3, 17, 18]. That functional capacity of sap should decline in diabetes suggests that the fall in starvation may relate to lower circulating insulin levels and indeed injection of insulin to normal animals raises the activity of the sap [19]. Conversely there may be mechanisms other than endocrine which influence rates of protein synthesis in both liver and muscle according to dietary state [20]. In the in vitro system used here the ribosomes are enriched with respect to the intracellular ratio of sap to ribosomes, which might influence how important such changes will be in vivo. Sap or pH 5 fraction is of course a mixture of components. If indeed fasting does not reduce the activity of amino acid activating enzymes [4], the change may lie in some aspect of the amino acid accepting capacity of the transfer-RNA [21] which could be related to changes in the isoaccepting forms as noted after hypophysectomy [22]. On the grounds that there is little initiation taking place in hepatic cell-free systems [23] it is unlikely that changing availability of initiation factors is chiefly responsible. With sap from diabetic muscle the defect appears to lie in aminoacyl-tRNA charging rather than the content of transferase [3]. In bacteria the sap fraction also appears to exercise a controlling influence, the presence of an inhibitor being implicated [24]. Further work is required to determine whether factors in mammalian sap claimed both to enhance and inhibit ribosome activity [25, 26] are responsible for the present observations.

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