THE INSULIN-LIKE EFFECTS OF A GLUCOSE METABOLITE (bis-PEP) FROM MUSCLE AND ERYTHROCYTES

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Received 18 October 1976

1. Introduction

It has been previously demonstrated with rat diaphragm muscle [1] that insulin increases the formation of a glucose intermediate, which has been described as 1,2-bis-phosphoenol pyruvate (bis-PEP) [2,3], by about 60% in thirty seconds. Work to verify the chemical structure and to study the effect of this high energy metabolite on the metabolism of tissues has now become possible by the preparation of this compound in greater quantities from bovine erythrocytes. Further work on its chemical structure is in progress; for convenience it is referred to as bis-PEP in this paper. In the present communication it is shown that bis-PEP significantly stimulates the incorporation of [^{14}C]glucose into glycogen in rat diaphragm muscle, the oxidation of [1-^{14}C]glucose by rat epididymal fat pad and the synthesis of fatty acids from [^{14}C]glucose and tritiated water in mice parametrial or mesenteric adipose tissue.

2. Materials and methods

2.1. *Experimental animals*

Normally fed crossbred Wistar rats of 100–125 g were used. Female mice were obtained from a random-bred closed colony bred at Imperial College [4].

2.2. *Materials*

[U-^{14}C]glucose, [1-^{14}C]glucose and $^{3}H_2O$ were obtained from Amersham. Insulin (glucagon-free) was obtained from Burroughs Wellcome, other chemicals were of the highest grade commercially available.

2.3. *Preparation of bis-PEP*

Bis-PEP was isolated from bovine erythrocytes by column-chromatography on Dowex 1 X 8 in Cl-form, using a variation of the elution scheme of Khym and Cohn [6] as described previously [7]. As shown in fig.1, the same elution pattern is obtained whether bis-PEP is isolated from diaphragms or erythrocytes. The identity of the preparation from erythrocytes and diaphragm has been further ascertained by comparison of infrared spectra and of P:C ratios. Also co-chromatography of ^{3}H-labelled bis-PEP from erythrocytes with the ^{14}C-labelled preparation from diaphragm allowed identification on high pressure columns as described by Bessman [8] fig.2, bis-PEP being prepared as described below.

Bis-PEP from erythrocytes used in these experiments was prepared directly from the Dowex column eluates, concentrated by shaking with iso-butanol and drying the waterphase.

This preparation contained ammonium chloride and potassium tetraborate and some ninhydrin-positive material. It is contaminated with glucose-6-phosphate in amounts usually too small for enzymic determination in the eluate of the Dowex column, but clearly demonstrable on the high pressure column. Salts were removed by passing the column eluate, taken up in 1 ml 1 M HCl, over a Sephadex G-10 column and drying under N_2. Calcium chloride, when indicated, was added to stabilize the compound and

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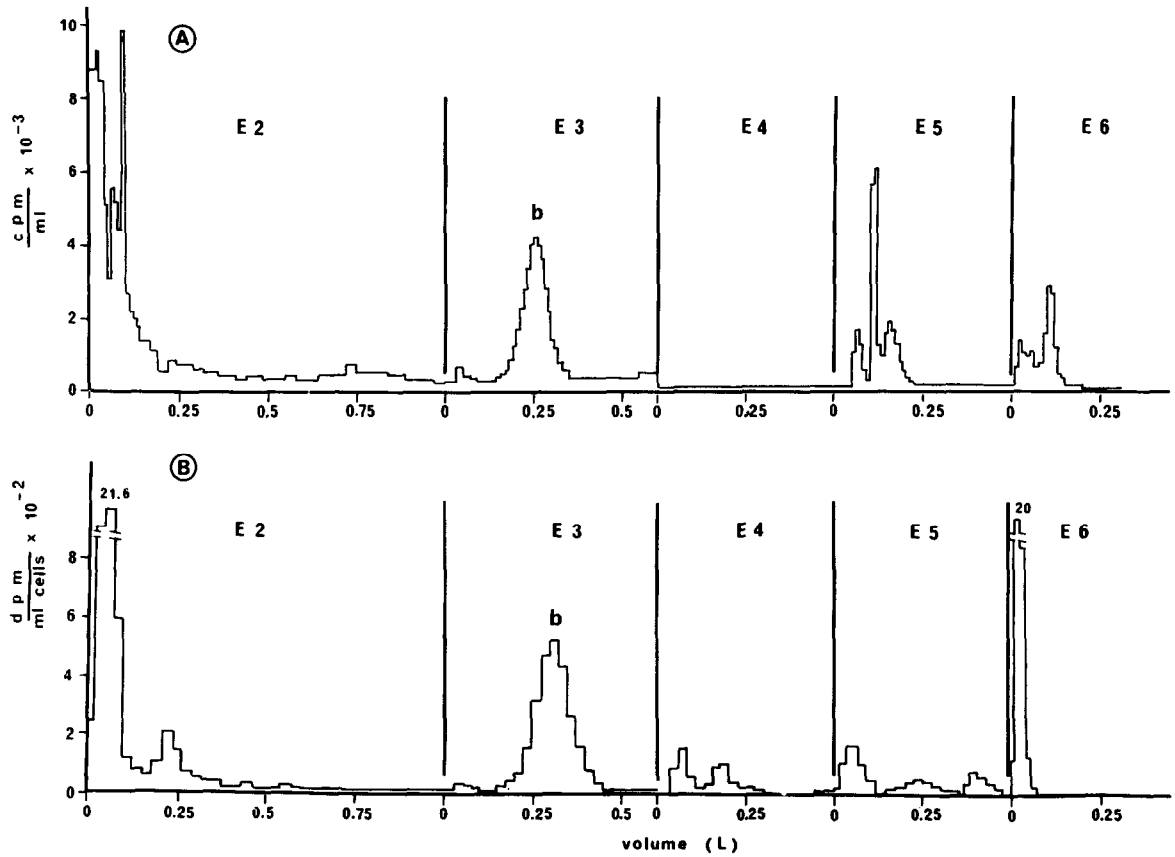

Fig.1. Comparison of elution pattern of [^{14}C]glucose metabolites from hemolysed erythrocytes and diaphragm incubation media. Resin: Dowex 1 X 8200–400 mesh, column 15 X ϕ1 cm, flow rate 1 ml/min. Ⓐ Diaphragm incubation medium, Ⓑ Hemolysate. E_3 contains glucose-6-phosphate and bis-PEP (overlapping, marked b).

the final preparation contained known amounts of this salt.

The amount of bis-PEP used in the experiments was determined by total dpm and/or determination of total P, assuming no dilution of the specific activity of glucose calculating a P:C ratio of 2:3. Glucose-6-phosphate is known to play no role in the stimulating

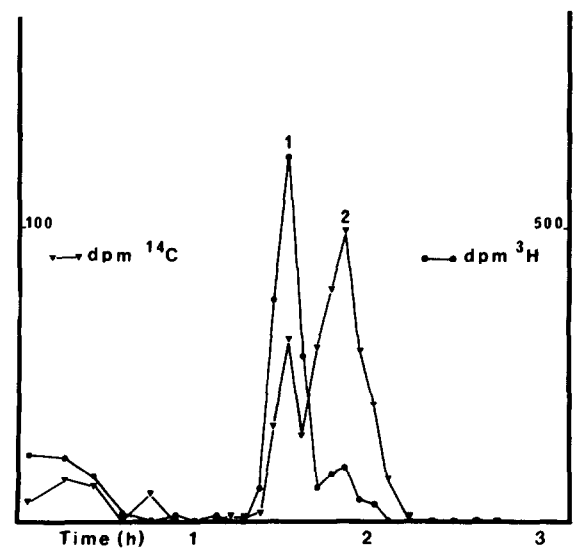

Fig.2. High pressure chromatography of [^{14}C]labelled bis-PEP from rat diaphragm mixed with (^{3}H)-labelled bis-PEP from bovine erythrocytes. Resin: Aminex A 25/Durrum X4 (2:1), linear gradient 0.1–0.4 M NH_4Cl in 0.05 M $K_2B_4O_7$. Pressure 200 psi, flow rate 0.2 ml/min, column cm X ϕ3 mm. 1. Bis-PEP. 2. Glucose-6-phosphate. This batch of diaphragm medium contained an unusually high amount of glucose-6-phosphate.

effect of insulin on glycogen synthesis, nor has it any stimulatory effect of its own on glycogen synthesis, in the concentrations used in these experiments [9].

Some control experiments with glucose-6-phosphate separated from bis-PEP in the high pressure column have been performed on epididymal fat pads. No effects could be demonstrated. To ascertain that the effects described in this paper were due to the presence of bis-PEP and not to some contaminant, the activity of a batch of bis-PEP on the synthesis of fatty acids in mouse adipose tissue was determined before and after destruction of bis-PEP by freeze drying. After freeze-drying no stimulation of fatty acid synthesis could be demonstrated.

2.4. *Incubation techniques*

2.4.1. Rat

The diaphragm incubation technique was as previously described [5]. Hemidiaphragms were preincubated for 10 min in 2 ml buffer solution containing 0.5 μCi [U-^{14}C]glucose/ml, then 0.4 ml of buffer containing bis-PEP to give a final concentration of 15 or 2 nmol/ml or an equivalent amount of $CaCl_2$ for the controls, were tipped in from the side arm. The oxygen consumption was measured throughout, as a control on the condition of the tissue during incubation. Pairs showing a difference of more than 10% were rejected.

Pieces of epididymal fat pad weighing approximately 150 mg were incubated at 37°C in a Dubnoff shaker in 4 ml Krebs-Ringer bicarbonate 2% (w/v) bovine albumin, and 0.25 μCi [1-^{14}C]glucose/ml with or without 10 nmol bis-PEP/ml. The glucose concentration was 0.2% (w/v), insulin concentration, was 25 μU/ml.

After 30 min incubation 0.15 ml hyamine was injected into cups in the centre wells, and 0.6 ml 4 M HCl into the media. Incubation continued for another 30 min.

2.4.2. Mice

Pieces of parametrial or mesenteric adipose tissue weighing 20–40 mg were incubated in Krebs-Ringer bicarbonate at 37°C for 30 min in a shaking water bath. The volume of the medium was 2 ml and contained 2% (w/v) defatted bovine serum albumin, 15 μmol glucose/ml, 0.2 μCi [U-^{14}C]glucose/ml, 0.4 mCi ^3H_2O/ml with or without bis-PEP at 15 or 1.5 nmol/ml. Insulin was 0.25 mU/ml. Control flasks contained an equivalent amount of $CaCl_2$.

2.5. *Analytical methods*

2.5.1. Estimation of tissue glycogen

Tissue glycogen was extracted and prepared for determination as described previously [5], except that the tissue was frozen in liquid nitrogen and pulverized before adding trichloroacetic acid. Glycogen prepared from rat livers was used for co-precipitation.

Purified precipitates were taken up in 1 ml of water, 0.3 ml samples being used for liquid scintillation counting using the channels ratio quench correction technique. All samples were taken in triplicate.

2.5.2. Fatty acid synthesis

Adipose tissue pieces were directly saponified in

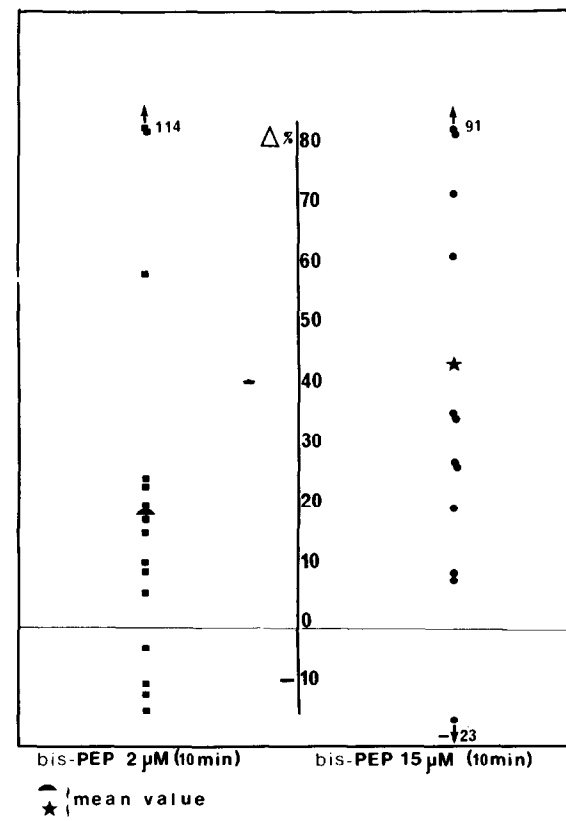

Fig.3. Influence of bis-PEP in incubation medium on the incorporation of [^{14}C]glucose into free glycogen of diaphragm pairs. Δ%: Percentage increase in dpm of free glycogen from bis-PEP treated halves over controls.

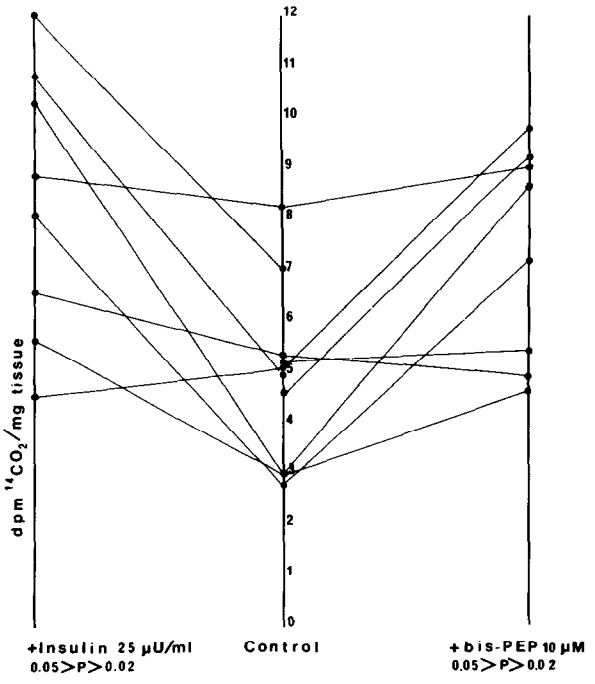

Fig.4. Influence of bis-PEP on the oxidation of C_1 from glucose in rat epididymal fat pads.

ethanolic potassium hydroxide and the fatty acids extracted with petroleum ether (40–60°C) after acidification. The ^{14}C- and ^{3}H-content was determined by liquid scintillation counting as previously described [9].

3. Results and discussion

The results given in fig.3 are expressed as percentage increase over the paired control although the P values were calculated on actual values. As appears from fig.3 incubation of rat diaphragms with 2 or 15 nmol/ml of bis-PEP caused a significant increase in the incorporation of [^{14}C]glucose into free glycogen within 10 min. The increase in the 2 nmol/ml group by itself is of borderline significance ($0.1>P>0.05$), but with 15 nmol/ml it is significant ($0.05>P>0.02$). In the series as a whole the effect is highly significant: $P<<0.01$. The influence of bis-PEP on the oxidation of C_1 from glucose by epididymal fat is highly significant ($0.05>P>0.02$) (fig.4) and of the same order of

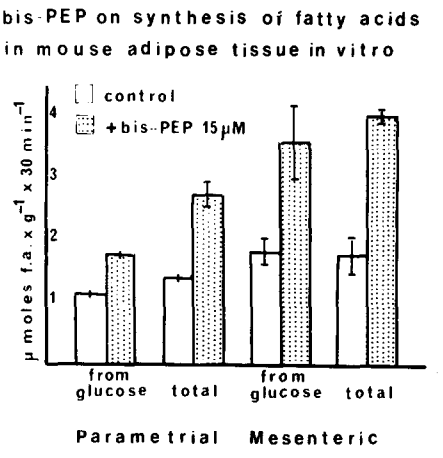

Fig.5. Effect of bis-PEP on fatty acid synthesis in adipose tissue in vitro from mice aged 10 weeks. Means of 3 experiments for each column, vertical bars indicate S.E.

magnitude as the effect of insulin in the concentrations used.

Bis-PEP at a concentration of 15 nmol/ml stimulates the rate of glucose incorporation into fatty acids and the total rate of fatty acid synthesis in mouse parametrial and mesenteric adipose tissue (fig.5,6). This stimulation is significant ($P<0.05$) and of the same order as that produced by insulin (fig.6). At the lower concentration of 1.5 nmol/ml, the stimulation by bis-PEP is not significant.

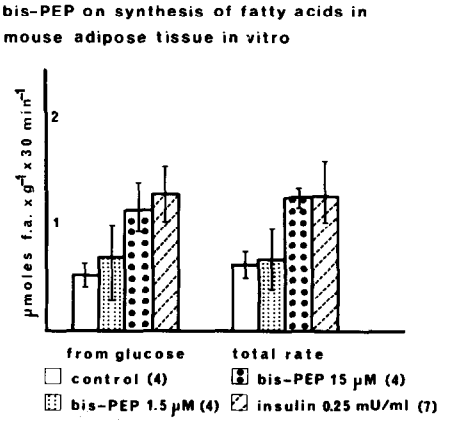

Fig.6. Effect of bis-PEP on fatty acid synthesis, in adipose tissue in vitro from mice aged 3–4 months. 14 mM $CaCl_2$ was present in bis-PEP-and control media. No. of experiments is given in brackets, vertical bars indicate S.E.

In the presence of bis-PEP at the higher concentration, the glucose contribution to the total rate of fatty acid synthesis is lower than in the presence of insulin, and this may be due to the bis-PEP acting as a substrate for fatty acid synthesis.

The results reported in the present communication show that the presence of μmolar concentrations of bis-PEP in vitro exert insulin-like effects on the incorporation of glucose into glycogen in diaphragm, on the oxidation of glucose via the phosphogluconate pathway in epididymal fat and on the synthesis of fatty acids as measured with tritiated water as well as on fatty acid synthesis from glucose, in parametrial and mesenteric adipose tissue. These results are compatible with the hypothesis that the rapid increase of bis-PEP under the influence of insulin might play a part in translating the insulin stimulus into effects on intracellular metabolism.

Acknowledgement

We are grateful to the Wellcome Trust for a grant which supported the work at Imperial College.

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