Vol. 93, No. 2, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
March 28, 1980 Pages 617-624

MEASUREMENT OF PHOSPHATE ESTERS IN EXTRACTS OF FETAL RAT HEART BY AUTOMATED HIGH PRESSURE LIQUID CHROMATOGRAPHY

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Received February 4,1980

SUMMARY: Creatine phosphate, nucleotides and glycolytic phosphate esters were estimated in extracts of beating, in situ freeze clamped, 13 1/2 to 19 1/2 day fetal rat hearts by automated phosphate ester chromatography. Creatine phosphate increased more than 4-fold to almost 9 n moles per mg. protein at 19 1/2 days, while ATP remained relatively constant at about 19 to 21 n moles per mg. protein. Most other nucleotides decreased as gestation advanced. ATP rather than creatine phosphate appears to be the major energy source of fetal rat heart. Except for glucose-6-phosphate, which increased, the glycolytic phosphate esters decreased only very slightly with advancing gestational age, suggesting a relatively stable basal glycolytic activity. Methodology includes correction for phosphate esters of whole blood trapped in extracts of in situ freeze clamped tissues.

INTRODUCTION: High energy phosphate compounds and phosphorylated glycolytic intermediates were measured simultaneously in extracts of beating, in situ freeze clamped fetal rat heart from days 13 1/2 to 19 1/2 of gestation by means of an automated phosphate analyzer (1, 2, 3) which combines column chromatography and an automated fraction collector and ashing machine, and is sufficiently sensitive to detect as little as 0.15 n moles of an individual phosphate ester in extracts prepared from the equivalent of 1 mg of tissue protein. The quantities of the phosphorylated compounds estimated compare favorably with individual measurements of fetal rat heart using enzymatic analyses (4, 5, 6).

METHODS:
Fetal heart extracts: Timed pregnant, albino rats (Simonson, Gilroy, CA) were anesthetized by i.p. injection of 40 mg./kg. sodium pentobarbital, and the fetuses exposed in situ with umbilical circulation intact. The heart was exposed surgically, freeze clamped between the ends of blunt forceps cooled in liquid nitrogen, and dropped into liquid nitrogen; only beating hearts were collected. Pooled hearts were pulverized in a stainless steel impact mortar and extracted with 0.6M $HClO_4$ (7), the precipitated protein being reserved for analysis (8). The neutralized extracts were stored at -70°C; they usually measured from 0.5 to 0.75 ml. in volume, and represented the equivalent of 1 to 5 mg. of protein. An entire extract was used to obtain a chromatographic analysis of the phosphates it contained.
Chromatography: The fetal rat heart extract was applied to a 0.3 x 15 cm precolumn containing about 2 cm of AG MP-1 resin (BioRad Labs., Richmond, CA) topped with a small amount of Celite 545 (Supelco, Inc., Bellefonte, PA). The precolumn was attached to a 0.3 x 50 cm analytical column packed with a 1:1 w/w mixture of high efficiency anion exchange resins, Aminex A-25 (BioRad Labs.) and DA-X4 (Durrum Chemical Co., Palo Alto,

Abbreviations: CP = creatine phosphate; CPK = creatine phosphokinase; G-6-P = glucose-6-phosphate; F-6-P = fructose-6-phosphate; F-1,6-P_2 = fructose-1,6-diphosphate; G-1,6-P_2 = glucose-1,6-diphosphate; DHAP = dihydroxy acetone phosphate; GA-3-P = glyceraldehyde-3-phosphate; G-1-P = glucose-1-phosphate; 3-PGA = 3-phosphoglyceric acid; S-7-P = sedoheptulose-7-phosphate; M-6-P = mannose-6-phosphate; 2,3-DPG = 2,3-diphosphoglyceric acid; α-GP = α-glycerophosphate.

0006-291X/80/060617-08$01.00/0

CA). Any space in the precolumn was filled with water adjusted to pH 9.0, and the precolumn was attached to a high pressure pump and gradient reservoir. Elution speed was set at about 0.2 ml per minute, and elution of the phosphate compounds accomplished by using an increasing linear gradient of ammonium chloride in constant ammonium tetraborate solution. The effluent samples were fed into silicon cups in the rotating table of the phosphate analyzer, evaporated to dryness and ashed in a nitric acid-potassium borate mixture. The dry ash was dissolved in dilute sulfuric acid and subjected to molybdenum blue color development which was monitored by a flow colorimeter connected to an integrating recorder, and also to a PDP-11 computer programmed to collect data.
Because of the extremely small size of the 13 1/2 and 14 1/2 day fetal hearts, and the difficulty in blotting dry these minute semi-gelatinous specimens for accurate weight determination (see ref. 9 for difficulties inherent in blotting dry adult rat atrial specimens), we have expressed all measurements on a per mg. protein basis, i.e. as n moles/mg.

RESULTS AND DISCUSSION: A typical chromatogram of a 13 1/2 day fetal heart extract is seen in Fig. 1, where the phosphate compounds are numbered in the order in which they elute from the column. Peaks A and B represent phospholipid precursors whose identification and quantification are the subject of other studies (10). Area A contains glycerol-3-phosphorylcholine and glycerol-3-phosphorylethanolamine, and area B contains phosphocholine, phosphoethanolamine and other compounds. Peaks designated #1 through #24 have been identified by cochromatography with authentic compounds. Table I presents the content in n moles/mg. of phosphate compounds present in the extracts in amounts sufficient for quantification, i.e. at least 0.15 n moles/mg. A striking feature of this investigation is the very low CP content of the 13 1/2 day extracts compared with their ATP content, and the progressive increase in CP from 2.18 ± 0.08 n moles/mg. at 13 1/2 days to 8.81 ± 0.38 n moles/mg. at 19 1/2 days, about a 4-fold increase, while ATP remains relatively stable. In mouse heart it was found (11) that total creatine (creatine + CP) increases 7-fold between fetal days 13 to 15 and neonatal day 1. We found CP to be 15.87 ± 0.97 n moles/mg in extracts of newborn rat heart, a greater than 7-fold increase over the fetal 13 1/2 day extracts. It appears therefore, that immediately following birth the cardiac energy demands specifically require a large CP component, since the ATP of our newborn heart extracts was 22.76 ± 1.06 n moles/mg., close to the prenatal range of about 19 to 21 n moles/mg. Apparently the compartmentation of energy supply and demand in the prenatal rat heart is such that a relatively constant amount of ATP (around 20 n moles/mg. in our extracts) together with gradually increasing amounts of CP (which are relatively low) are geared to the cardiac energy requirements between days 13 1/2 and 19 1/2 of gestation. These findings suggest that the creatine-CP shuttle (12, 13) becomes the predominant mechanism of energy transport around the time of birth. It has been shown in fetal mouse heart (14) that total CPK activity increases 8.5-fold from day 14 of gestation to term, due principally to the MM-CPK isoenzyme. A marked increase in the MM-CPK isoenzyme has been demonstrated (15) in fetal pig heart coincident with increasing late gestation age. Controlling factors regulating ATP and CP content of fetal rat heart, and changes imposed thereon at birth are currently under investigation in this laboratory. The ATP content of the extracts increased from 17.70 ± 0.67 n moles/mg. at 13 1/2 days to 18.89 ± 0.76

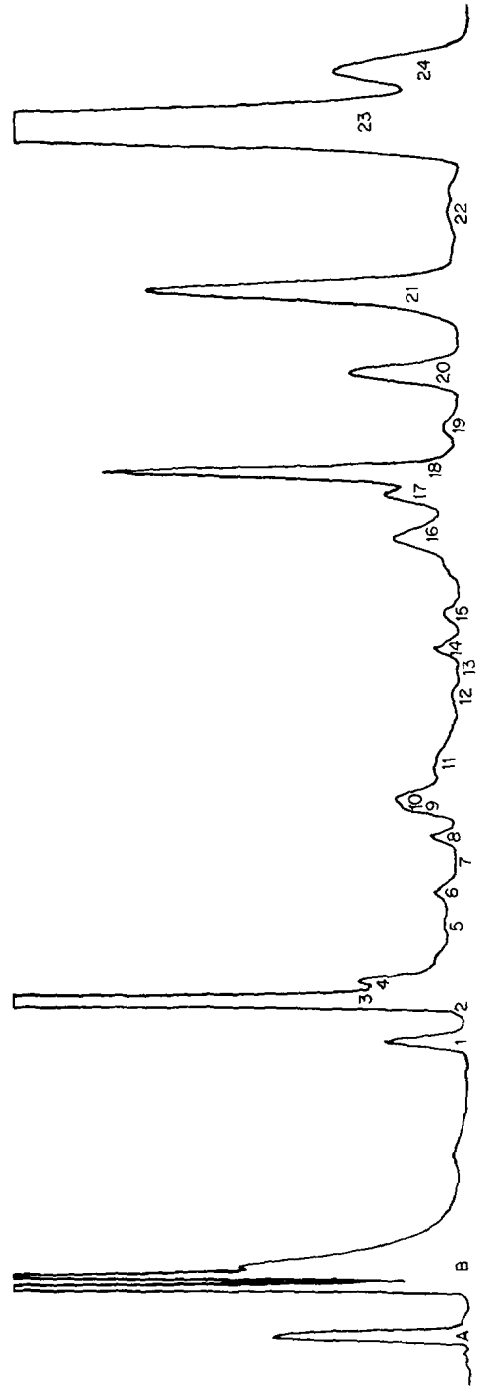

Fig. 1. Chromatogram of an extract (containing equivalent of 3.45 mg. protein) of 13 1/2 day fetal hearts pooled from 2 litters.

Peak identification: A and B = phospholipid precursors; 1 = creatine phosphate; 2 = glucose-1-phosphate; 3 = inorganic phosphate; 4 = α-glycerophosphate; 5 = dihydroxy acetone phosphate; 6 = mannose-6-phosphate; 7 = glyceraldehyde-3-phosphate; 8 = fructose-6-phosphate; 9 = sedoheptulose-7-phosphate; 10 = glucose-6-phosphate; 11 = nicotinamide adenine dinucleotide; 12 = pyrophosphate; 13 = 3-phosphoglyceric acid; 14 = uridine-5'-monophosphate; 15 = glucose-1,6-diphosphate; 16 = adenosine-5'-monophosphate; 17 = fructose-1,6-diphosphate; 18 = 2,3-diphosphoglyceric acid; 19 = uridine-5'-diphosphate + inosine-5'-monophosphate; 20 = cytidine-5'-triphosphate; 21 = adenosine-5'-diphosphate + uridine-5'-triphosphate; 22 = guanosine-5'-diphosphate; 23 = adenosine-5'-triphosphate; 24 = guanosine-5'-triphosphate.

Table I. Phosphate esters of extracts of fetal rat heart expressed in n moles/mg. protein as the mean ± SEM.

Fetal Age	13 1/2 day	14 1/2 day	15 1/2 day	16 1/2 day	17 1/2 day	18 1/2 day	19 1/2 day
Number of analyses	10	6	6	4	4	6	6
CP	2.18±0.08	1.89±0.17	2.00±0.20	2.94±0.65	4.11±0.36	7.37±0.47	8.81±0.38
ATP	17.70±0.67	18.89±0.76	19.73±0.59	20.32±0.70	19.57±0.70	19.90±0.75	20.84±1.34
ADP	7.90±0.39	7.63±0.44	6.64±0.41	6.30±0.33	5.85±0.38	5.98±0.58	5.83±0.14
AMP	4.35±0.29	3.28±0.30	2.95±0.14	2.34±0.17	2.24±0.16	2.43±0.32	2.08±0.16
CTP	1.42±0.07	1.30±0.07	1.25±0.07	1.33±0.18	1.13±0.04	1.05±0.04	0.95±0.02
UMP	0.64±0.05	0.43±0.02	0.42±0.05	0.48±0.08	0.30±0.02	0.39±0.09	0.23±0.08
UDP + IMP	0.85±0.01	0.52±0.05	0.93±0.15	0.73±0.04	0.30±0.04	0.29±0.09	0.43±0.08
GDP	0.72±0.09	0.54±0.09	0.51±0.02	0.54±0.04	0.48±0.06	0.44±0.04	0.59±0.07
GTP	2.67±0.29	2.37±0.08	2.19±0.06	2.49±0.18	2.87±0.07	2.31±0.25	2.23±0.15
NAD	1.52±0.22	1.36±0.09	1.44±0.23	1.32±0.39	1.41±0.09	1.68±0.16	1.32±0.10
G-6-P	2.07±0.15	2.18±0.19	2.39±0.17	2.57±0.20	2.48±0.21	2.82±0.24	2.62±0.04
F-6-P	0.76±0.12	0.61±0.04	0.57±0.16	0.68±0.02	0.56±0.09	0.66±0.12	0.59±0.11
F-1,6-P_2	1.68±0.11	1.61±0.17	1.41±0.32	1.41±0.16	1.24±0.08	1.25±0.03	1.18±0.07
G-1,6-P_2	0.23±0.01	0.22±0.03	0.16±0.01	0.21±0.01	0.19±0.01	0.22±0.01	0.16±0.03
S-7-P	1.18±0.11	1.03±0.14	neg.	neg.	neg.	neg.	neg.
DHAP	0.62±0.11	0.52±0.06	0.96±0.09*	0.91±0.08*	0.77±0.07*	1.07±0.20*	1.04±0.02*
M-6-P	0.69±0.12	0.46±0.04					
2,3-DPG	3.32±0.32	3.31±0.29	2.93±0.66	1.82±0.38	1.12±0.09	0.54±0.07	0.46±0.06
Pi	31.68±2.94	29.82±2.67	30.10±2.73	26.54±2.87	24.36±1.98	22.61±0.97	22.01±0.35
adenylate energy charge**	0.722±0.008	0.766±0.006	0.787±0.013	0.811±0.011	0.811±0.010	0.820±0.008	0.820±0.002

Pooled hearts from 2 litters were used for 13 1/2 and 14 1/2 day extracts; pooled hearts from 1 litter were used for 15 1/2 and 16 1/2 day extracts; three 18 1/2 day hearts and 2 19 1/2 day hearts were combined to make an extract for a single chromatogram.
neg. = negligible quantity, i.e. peak too small for accurate quantification.
* = after 14 1/2 days the sum of DHAP + M-6-P is given
** = adenylate energy charge : $\frac{1}{2}\ \frac{ADP + 2\,ATP}{AMP + ADP + ATP}$

n moles/mg. at 14 1/2 days, and thereafter remained in a relatively stable range of about 19 to 21 n moles/mg. throughout the remainder of the gestation period studied. The ADP content of the extracts decreased from 7.90 ± 0.30 n moles/mg. at 13 1/2 days to 5.85 ± 0.38 n moles/mg. at 17 1/2 days, and remained essentially at this level through the next two days. The figures presented for ADP are somewhat higher than the actual amounts of ADP, since UTP separates under the ADP peak in our chromatogram. We are currently developing a correction for the UTP contribution. The AMP content of the extracts decreased from 4.35 ± 0.29 n moles/mg. at 13 1/2 days to 2.08 ± 0.16 n moles/mg. at 19 1/2 days. In general, most of the nucleotides we were able to quantify except ATP, decreased in the extracts as gestation advanced. The most abundant of the non-adenine nucleotides was GTP which decreased only very slightly, remaining about 2.45 n moles/mg., which may reflect a stable requirement for protein synthesis during the period of gestation studied. GDP presented as a broad, rather flat peak, which was difficult to quantify with the same accuracy as GTP; its content in the extracts appeared to decrease as gestation advanced. CTP presented as a very sharply defined and symmetrical peak, and decreased from 1.42 ± 0.07 n moles/mg. at 13 1/2 days to 0.95 ± 0.02 n moles/mg. at 19 1/2 days. The other nucleotides we could quantify, UMP and UDP + IMP, were present in amounts less than 1.0 n moles/mg. in the 13 1/2 day extracts, and decreased with advancing gestational age. The NAD peak was difficult to quantify with accuracy; however, the data suggested that the NAD content of the extracts did not change markedly with advancing gestational age, remaining close to 1.43 n moles/mg.

Traditionally it has been claimed that glycolysis becomes progressively less important than aerobic metabolism in fetal heart as gestation proceeds (16, 17, 18, 19, 20). The actual amounts of glycolytic intermediates we measured in the extracts indicate, however, that fetal heart glycolysis decreases only very slightly during days 13 1/2 to 19 1/2. This suggests that there is a relatively constant basal glycolytic activity during fetal cardiac development, which agrees with studies of glycolytic enzyme activity in newborn versus adult rat heart (21), and that any increasing aerobic metabolism is superimposed upon, rather than replaces, the glycolytic component. In the fetal heart extracts G-6-P was the most abundant glycolytic intermediate (possibly reflecting the use of glucose as a major fetal fuel (22)), and increased slightly as gestation advanced, i.e. from 2.07 ± 0.15 n moles/mg. at 13 1/2 days to 2.62 ± 0.04 n moles/mg. at 19 1/2 days. Over this time span, the extracts contained decreasing amounts of F-6-P, F-1,6-P_2 and S-7-P, suggesting a control over the amount of G-6-P directed through the glycolytic and pentose pathways as gestation advanced. Another study of glycolytic intermediates in fetal rat heart (6), showed active glycolysis in the three fetal ages studied (sic. 16 1/2, 20 1/2 and 21 1/2 days), and an increase in G-6-P, F-6-P and F-1,6-P_2 in 20 1/2 day heart compared with 16 1/2 day. The amount of G-1,6-P_2 in our extracts remained about 0.20 n moles/mg. throughout the period we studied. In extracts of 13 1/2 day and 14 1/2 day hearts, DHAP

measured less than 1.0 n moles/mg. and separated close to the M-6-P peak which estimated approximately equal to the DHAP peak. With advancing gestational age, the combined DHAP + M-6-P peaks measured about 1.0 n moles/mg. In chromatograms of extracts of fetal hearts from all the gestational ages there was no evidence of a peak in the anticipated position (#7 in Fig. 1) for GA-3-P, which therefore must have measured less than 0.15 n moles/mg. at all ages studied. The extracts contained appreciable amounts of α-GP (for example, 1.83 ± 0.21 at 13 1/2 days and 2.31 ± 0.49 at 14 1/2 days) but the separation was not consistently of the quality to permit reliable quantification at all ages. The very minute G-1-P peak rarely separated distinctly.

The glycolytic intermediate 2,3-DPG separated clearly and consistently especially in the chromatograms of the younger fetal hearts. Since this substance is considered to occur as a major metabolite only in red blood cells, its presence in the extracts indicated they contained fetal blood. Accordingly, the chromatograms and the estimated quantities of intermediates presented in Table I are more correctly described as amount per mg. sum of cardiac muscle protein plus whole blood protein, and represent the phosphate esters in fetal rat heart plus fetal blood trapped by freeze clamping. This would explain differences in some phosphate esters and inorganic phosphate measured by us and by others who used washed fetal rat heart (5,6), which would contain essentially no blood. The automated phosphate analyzer can be used to make an approximate correction for blood contamination, which can be applied to other studies using in vivo freeze clamping of beating adult heart, liver and lung etc. A detailed report of such a study is in preparation. Briefly, whole blood is collected directly from in situ beating fetal hearts, added directly to 0°C 0.6M $HClO_4$, and extracted and chromatogrammed as described in Methods. Relatively large quantities of 2,3-DPG are present in the whole blood of the youngest fetuses from which were able to collect enough blood for extraction and chromatography, i.e. at 14 1/2 days, where the 2,3-DPG was 8.37 ± 0.21 n moles /mg. With advancing age the 2,3-DPG of fetal whole blood dropped progressively to 0.75 ± 0.01 n moles/mg. at 19 1/2 days, reached a minimum value of 0.305 ± 0.06 n moles/mg. at 21 1/2 days and began to rise immediately at birth. Assuming that all the 2,3-DPG of the fetal heart extracts comes from fetal whole blood, an approximate correction for the whole blood phosphate compounds can be made, as illustrated in Table II for extracts of 14 1/2 day hearts. The amounts of phosphate compounds present in 14 1/2 day fetal whole blood are listed in column II, and column V lists the calculated amounts in fetal cardiac muscle after correcting for the blood phosphates. Unfortunately, we were not able to apply this correction method to fetal heart extracts from all gestational ages studied, because the small quantities of 2,3-DPG in older fetal blood presented in fetal heart chromatograms as a small peak not delineated sharply enough to be used as a criterion for calculating how much fetal blood was present in the extracts of late fetal heart. On the other hand, since adult blood contains large amounts of 2,3-DPG (10.14± 0.43 n moles/mg. in adult female rat whole blood extracts), the 2,3-

Table II. Approximate corrections for presence of 14 1/2 day fetal whole blood phosphates in extracts of 14 1/2 day fetal heart.

	I	II	III	IV	V
	n moles/mg.protein cardiac muscle + whole blood*	n moles/mg.protein whole blood	calculated n moles/ 0.374 mg. protein whole blood**	calculated n moles/ 0.626 mg. protein cardiac muscle ***	calculated n moles/ mg. protein cardiac muscle****
2,3-DPG	3.13	8.375	3.13	0.0	0.0
CP	1.89	0.41	0.15	1.74	2.78
ATP	18.89	7.19	2.69	16.20	25.88
ADP	7.63	3.39	1.27	6.36	10.16
AMP	3.28	1.20	0.45	2.73	4.36
CTP	1.30	0.55	0.20	1.10	1.76
UMP	0.43	neg.	-	0.43	0.68
UDP + IMP	0.52	neg.	-	0.52	0.83
GDP	0.54	neg.	-	0.54	0.86
GTP	2.37	0.71	0.26	2.11	3.37
NAD	1.36	0.43	0.16	1.20	1.92
G-6-P	2.18	0.87	0.32	1.86	2.97
F-6-P	0.61	neg.	-	0.61	0.97
F-1,6-P_2	1.61	0.59	0.22	1.39	2.22
G-1,6-P_2	0.22	neg.	-	0.22	0.35
S-7-P	1.03	0.89	0.33	0.70	1.12
DHAP + M-6-P	0.98	0.77	0.29	0.69	1.10
Pi	29.82	26.00	9.72	20.10	32.11

* = values from Table I.

** = assuming that the 3.13 n moles of 2,3-DPG / 1 mg.protein in 14 1/2 day heart extract represents 0.374 mg. whole blood, since 8.375 n moles of 2,3-DPG represent 1 mg. whole blood protein (column II).

*** = assuming that 1 mg. protein 14 1/2 day fetal heart extract represents 0.374 mg. whole blood protein plus 0.626 mg. cardiac muscle protein; therefore values in column IV are calculated as the difference between amounts in column I and column III.

**** = values calculated from column IV.

neg. = negligible quantity.

DPG peak in chromatograms of adult tissues can serve as a reliable index of the amount of whole blood contamination in extracts of adult tissues that have been freeze clamped in vivo.

ACKNOWLEDGMENT: The generous contribution of time spent in consultation by Dr. Paul J. Geiger is gratefully acknowledged. We are especially indebted to Mrs. Catherine Rokaw for expert technical assistance. This work was supported in part by USPH Grant #HD09052, and in part by the Glycolysis Fund of the University of Southern California made possible by the generous contributions of the David and Minnie Berk Foundation and many individual donors.

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