Establishing A Baseline Fetal Ovine Plasma ACTH Value Does Not Require Frequent Sampling: Absence Of Significant Peaks Of Fetal ACTH Concentration In Plasma In Late Gestation

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BU-1222-M

September 1993

Submitted 9/1/93
Am. J. Ob. Gynecol.

ESTABLISHING A BASELINE FETAL OVINE PLASMA ACTH VALUE DOES NOT REQUIRE FREQUENT SAMPLING: ABSENCE OF SIGNIFICANT PEAKS OF FETAL ACTH CONCENTRATION IN PLASMA IN LATE GESTATION

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1 **CONDENSATION**

2 Baseline fetal ovine plasma ACTH varies during the 24h day but this variability does
3 not change with gestational age and is not composed of measurable discrete pulses.

4 **Key Words: fetus, sheep, adrenocorticotropin, pulsatility**

ABSTRACT

Objective: Fetal plasma ACTH may show short-term temporal variability. Our objective was to determine whether one fetal ovine plasma sample provides adequate information to establish a baseline fetal plasma ACTH concentration. If true, the ability to establish baselines with a single sample would greatly reduce the amount of plasma removed from a fetus to determine the baseline for an experiment.

Study design: We sampled plasma from four non-stressed fetuses at four different sampling rates. Four different gestational age windows were used to investigate age-related changes. Ewes were instrumented at 118 days gestational age (dGA). Fetuses were sampled at 125-129, 130-134, 135-139, 140-146 dGA. Twelve consecutive fetal carotid samples were withdrawn at each age at four sampling rates; every two, five, ten, and thirty minutes.

Mean, standard deviation, and coefficient of variation (CV) were determined for each 12 sample series. Gestational age increases in mean plasma ACTH were analyzed by linear regression of the four age groups. The CV's of the sampling rates, combined from all age groups, were compared by two way ANOVA, with the individual sheep as the second factor. Bonferroni's test of multiple comparisons was performed where necessary. The CV's of the four gestational age windows, combined from all sampling rates, were similarly compared. The presence of pulses were examined using the PULSAR program.

Results: Fetal blood gases were within normal limits for all time points. The mean regression equation was ACTH = 1.55 (dGA) - 178 $pg.ml^{-1}$, and was significant at the p = 0.05 level. The CV's were as follows: 125 dGA (48.8,25.1,8.1,8.5); 130 dGA (16.4,8.6,13.8, 34.1); 135 dGA (13.0,16.3,18.6,17.9); and 140 dGA (10.3,9.4,23.6, and 25.7); for the 2, 5, 10, and 30 min sampling rates, respectively. None of the comparisons were significant. The few pulses of ACTH detected were primarily detected in the 30 min sampling rate series.

Conclusions: These results support previous studies that show that fetal plasma ACTH does rise gradually in late gestation. Baseline fetal ovine plasma ACTH varies over time, but this variability does not change with gestational age and is not composed of measurable discrete pulses of ACTH.

BACKGROUND

Fetal ovine corticotropin (ACTH) may be released in a pulsatile manner. Some evidence exists which suggests that the adult pituitary secretes ACTH as discrete pulses (*1*). Plasma ACTH concentrations have been shown to vary over a one hour period in late gestation fetal sheep (*2*). Fetal plasma cortisol levels also show considerable variation over a 90 minute period (*3*). These cortisol fluctuations in undisturbed sheep may reflect an underlying ACTH variability.

Norman *et al.* (1985) demonstrated that basal ACTH levels in the fetal sheep vary with gestational age, and that this basal level increases at a rate of 1 $pg.ml^{-1}.day^{-1}$ over the last three weeks of gestation (*2*). We have described a similar rate of increase in the fetal sheep (*4*). This slow and steady rise in fetal plasma ACTH could reflect a change in pulse amplitude or frequency of ACTH release.

The existence of pulses of ACTH in fetal sheep plasma would complicate the assessment of fetal plasma ACTH concentration under basal conditions made following a single measurement of ACTH. The detection of basal ACTH pulses would be complicated by the low magnitude of the reported variability (*2*). While the pituitary response to a stressor may involve the release of a single, large, "pulse" of ACTH, the basal ACTH plasma level would be more likely to be maintained by regularly occurring pulses of low amplitude. Sampling plasma ACTH at a rate faster than the pulse frequency should more readily reveal the pulses than would less frequent sampling. In addition, sampling at several different frequencies should eliminate any variability inherent in the process of repeated measures and avoid false pulse detection.

Various methods have been used to detect pulses using time-series hormone data (*1, 5,6,7*). The PULSAR program (*7*) uses a running mean of the hormone series being investigated as the baseline, and the size of the template used to tag hormone peaks is based on the variability of the radioimmunoassay used for the hormone being investigated. This method was employed in the present study to detect pulses of fetal plasma immunoreactive-ACTH under the four different sampling rates.

The four following hypotheses were investigated in this study: the fetal sheep in late

gestation secretes basal ACTH in a pulsatile manner; the variability of fetal plasma ACTH increases with increasing gestational age, reflecting the greater maturity of the pituitary; the variability remains sufficiently low that a single ACTH sample provides a useful measure of the basal ACTH level; and the mean basal fetal plasma ACTH concentration increases in late gestation.

MATERIALS AND METHODS

Care of Animals: Four Rambouillet and Dorset cross ewes of known gestational age were used in these studies. Each ewe carried one fetus. Animals were housed in metabolic cages and given free access to alfalfa cubes and water ad libitum. Lights were on for 16 hours each day beginning 0600. New York State College of Veterinary Medicine guidelines for the care and use of the sheep were followed. Experiments were conducted under an approved Cornell Institutional Animal Care and Use Committee protocol.

Surgery was conducted under halothane general anesthesia at 118 ± 2 days gestational age (dGA) (mean ± SD) using techniques described in detail previously (*8*). Ewes were instrumented with polyvinyl catheters placed in the jugular vein and carotid artery and with multistrand bipolar stainless steel wires (AS632; Cooner Sales Co. Inc., Chatsworth, CA) sewn 5 mm apart into the myometrium of the pregnant horn to record the myometrial electromyogram. Fetuses were instrumented with polyvinyl catheters inserted into the jugular vein and carotid artery (*8*). The myometrial electromyogram was recorded as previously described (*8*), and was used only to indicate established labor, at which time the sampling protocol was terminated and the fetus allowed to be born.

Blood Sampling: Maternal and fetal blood samples were drawn into sterile ice-water chilled syringes, centrifuged at 4°C and the plasma was immediately flash frozen in liquid nitrogen. Fetal and maternal blood gases were determined on 0.5 ml of blood in an ABL2 Acid Base Laboratory (Radiometer A/S, Copenhagen, Denmark) at 39°C. Fetal blood was handled aseptically and the erythrocytes were returned at the end of each sampling series.

Sampling Regimen: Fetuses were sampled in four age windows: 125-129, 130-134, 135-139, and 140-146 dGA. Twelve consecutive fetal carotid blood samples (0.7 ml) were withdrawn over 15 seconds at each of four sampling rates: one sample every two (2) minutes, one every five (5) minutes, one every ten (10) minutes, and one every thirty (30) minutes. At least two hours elapsed between sampling series. Two sets of sampling rates were performed on one day, and the other two were performed the following day. The order in which the four sampling protocols was carried out was randomized. Morning sampling began between 0900 and 1100h and afternoon sampling began between 1400 and 1700h.

Immunoreactive Corticotropin (ACTH) Radioimmunoassay: Plasma ACTH was measured by radioimmunoassay in unextracted plasma using a miniaturized version of the INC Star ACTH kit (Stillwater, MN, USA) validated specifically for use in fetal sheep plasma and previously described in detail (*9*). Plasma was thawed on ice and 50 μl plasma was incubated with rabbit anti-human ACTH(1-24) antiserum for 24 hours at 4 ° C, and then incubated with iodinated ACTH(1-24) for another 24 hours at 4 ° C. The bound fraction was precipitated with a goat anti-rabbit second antibody (30 minutes at room temperature, 20-22 ° C).

The standard curve was generated from human plasma standards of concentrations 5, 10, 20, 50, and 100 $pg.ml^{-1}$. Interassay coefficient of variation (CV) for a fetal sheep plasma pool (mean concentration 28.8 $pg.ml^{-1}$, n=9) was 12.5%, and for maternal pool (mean concentration 42.8 $pg.ml^{-1}$, n=8) was 8.2%. Intraassay CV were 6.1 and 3.0% respectively. The assay sensitivity, calculated as the 90% bound fraction over the free fraction (B/B0) limit of detection, was 5.0 $pg.ml^{-1}$ of sample.

PULSAR Program Parameters: One ACTH assay was devoted to various fetal and maternal ovine plasma pools which covered the range of the assay. Each pool was run in a series of ten samples and used to generate an assay variability curve. This curve determined the "Noise Units" portion of the PULSAR program; the determination of how much variability would be due to the assay variability, rather than to a physiological ACTH pulse.

The PULSAR program used amplitude and duration as the criteria for peak determination. A smaller amplitude was required for a pulse containing two points than for a peak containing only one point. This diminishing amplitude-criterion was continued out to five points. The amplitude of each of these amplitude-criteria was determined empirically, using two data sets. The first data set consisted of the series of ten replicates of the pools used above, and was used as a negative control where no pulses should be detected. The first pass through each series had the ten replicates in the original assay order. The second pass through each series had the individual replicates ordered in such a way as to simulate one pulse. The second data set served as the positive controls and consisted of samples from three fetuses used in another experimental protocol. Twelve fetal plasma samples were obtained at a sampling rate of five minutes, and were taken at several different gestational ages from each sheep.

Up to six iterations were allowed in the determination of the running baseline. The iteration method weights points composing a pulse so as to minimize their effect on the determination of the trend line composing the baseline. A pulse was split into two when the intervening points fell by one standard deviation unit (SDU) below that of the peak concentration, even though the intervening point may not reach the baseline concentration.

Statistics: Gestational age increases in mean plasma ACTH were analyzed by linear regression. The mean ACTH values for each 12-sample series, fifty-one points in total, was used to generate the mean regression equation. Individual linear regression for each sheep were also conducted, using the mean ACTH value from each sampling rate versus the actual dGA.

The coefficient of determination, R^2, was used as the measure of the variability in mean plasma ACTH due to the gestational age. Analysis of variance was conducted on the regression results, where the F-test ($\alpha = 0.05$) was used to determine whether the calculated slope was significantly different from zero.

The CV from each sampling rate within an age group was compared by two-way analysis of variance using the individual sheep as the second factor. Significance at the $p < 0.05$ level was then further analyzed by Bonferroni's test of multiple comparisons. The

analysis was conducted using all sampling rates combined. The CV's from each sampling rate were similarly compared across the combined gestational age groups.

The number of pulses per twelve sample series was analyzed similarly to that of the coefficients of variation. Two-way analysis of variation was conducted using both the gestational age and individual sheep analysis, and the sampling rate and individual sheep analysis. Data throughout are provided as mean ± S.D. and n = 4 unless otherwise noted.

RESULTS

Fetal Condition: Fetal blood gases were within normal limits for all time points. Fetal PO_2 was 18.8 ± 1.2 mm Hg at the start of the experiment, and had not changed at the time of its conclusion.

The twelve-sample series of samples did not show a rise in trend in the ACTH levels from beginning to end of the series. This observation indicates that the sampling procedure did not disturb the fetus significantly. All ewes underwent spontaneous delivery. All fetuses were born alive and in good condition.

Linear Regressions: The mean linear regression equation was ACTH = 1.55 (dGA) -178, $p<0.05$. This indicated an increase of 1.55 $pg.ml^{-1}$ in fetal plasma ACTH per increase of one day of gestational age during the period studied, 125-146 days gestation.

Table 1 gives the results of the individual regression equations. The variables presented are the slope, coefficient of determination R^2 (adjusted for the degrees of freedom), and the F-statistic. All individual fetal ACTH regressions were significant and fit a linear model. However, fetus 3006 demonstrated an increase in the slope between 130 and 135 dGA windows, suggesting that a more complex, nonlinear, model may be more appropriate for this fetus.

Coefficients of Variation of Fetal Plasma ACTH: Table 2 gives the mean plasma ACTH values of each time series for each animal. These values were used to generate the mean regression equation. As seen here, the mean fetal plasma ACTH values obtained from the four different sampling rates for one sheep over one gestational age window were not always identical. Figure 1 gives one representative example of the twelve

fetal plasma ACTH samples obtained from fetus 3006 over the 125-129 dGA window at each of the four sampling rates. This age window was selected to illustrate the basal variability present in fetal plasma ACTH as no pulses were present.

Table 3 gives the coefficients of variation for each of the four dGA windows at each of the four sampling rates. Missing values seen in Tables 2 and 3 arose from early problems with catheter blockages and the presence of active labor. Due to the incomplete block structure of the results, the interaction term by two-way analysis of variance could only be determined for the sampling rate and individual fetal factors, but not for the gestational age window and individual fetal factors. The interaction term was not significant at the $\alpha = 0.05$ level. None of the analysis of variance tests for the sampling rate, the gestational age window, and the individual fetal factors were significant.

PULSAR pulse detection results: For the determination of the "Noise Units" in the PULSAR program, a binomial regression curve was fit to a fetal ovine ACTH radioimmunoassay variability curve. To generate this curve, ACTH values of multiple replicates of seven different plasma pools and their serial dilutions, covering the range of 9 to 120 $pg.ml^{-1}$, were quantitated by the RIA and plotted with the mean of the pools on the abscissa and the standard deviation s of the pool replicates on the ordinate. The binomial regression curve was expressed as:

$$y = 1.21 + 0.0119x + 0.000818x^2.$$

The PULSAR program's amplitude-criteria, determined by using the two test data sets, for the fetal plasma ACTH model were as follows: for a one-point pulse, the amplitude-criterion was 2.8 assay standard deviation units (SDU) above the baseline ACTH concentration; for a two-point pulse, 2.3 SDU; for a three-point pulse, 1.8 SDU; for a four-point pulse, 1.5 SDU; and for a five-point pulse, 1.2 SDU. All of the components of the model were now established.

Nineteen peaks were detected in fifty-one twelve-sample series representing 122.2 hours (Table 4). This gives a basal rate of 1 plasma ACTH pulse per 6.43 hours in a late-gestation ovine fetus. Seven sample series had multiple pulses detected. Of the twenty pulses detected, half were from the thirty-minute sampling rate time series. Half of the

pulses were between one and two standard deviations greater than the mean of the sample series, which was calculated including the points composing the pulse.

The two-way analysis of variance of pulses detected was not significant in any comparison. The interaction between the sampling rate term and the individual sheep term was also not significant. The interaction between the gestational age window term and the individual sheep term could not be determined due to the incomplete block design.

DISCUSSION

The lack of increase in plasma ACTH values from the beginning to the end of each sampling series indicated that neither the sampling rate, nor the volume of blood removed, was causing a stress-release of ACTH. Thus, the fetal plasma ACTH results were believed to represent true basal secretion.

The rate of increase of 1.6 $pg.ml^{-1}$ in fetal plasma ACTH per increase of one day of gestational age is similar to the rate reported previously by others (*2*), as well as ourselves (*4*), and supports the idea that ACTH provides the drive for the fetal plasma cortisol rise which initiates parturition and organ maturation in this species.

The low coefficients of variation suggest that the variability in plasma ACTH concentrations is not large enough to warrant the removal of multiple plasma samples from the fetus for the determination of a single time-point value in baseline samples, at rest. The variability does not appear to change with sample frequency or with dGA. However, different means for the same gestational age window were obtained with each series of samples; the individual series being no more than three days apart. In contrast, fetal plasma cortisol time series coefficients of variation have been reported to change with gestation (*3*). Challis et al. (1981) reported that fetal plasma cortisol variability was larger (36%) on days 11 to 20 before labor initiation than the variability (15-19%) measured on days 21 to 30 and days 5 to 0 before labor (*3*). The current results would suggest that this observation is due to maturation in the adrenal cortex itself, rather than changes in ACTH secretion.

The possibility remains for ACTH to be released in a pulsatile fashion in the fetal sheep in response to a stressor. Of particular interest would be the effect of a chronic

stressor, where the repeatability of any ACTH pulses on some timed fashion could be measured.

The PULSAR results demonstrate that pulses do occur. However, the frequency was very low suggesting that the pulses detected may have been in response to some change in homeostasis rather than to maintain the basal levels. Reported ACTH half-lives of two to seven minutes in sheep (*10*) and rats (*11*) suggest that more frequent pulses would be required to maintain basal levels than the few demonstrated here. However, the assay used may not be sensitive enough to enable PULSAR to detect small repeated pulses. A study which would involve intracellular voltage recording from corticotropin cells in the adenohypophysis might indicate the mechanism of maintenance of basal levels of ACTH release in the ovine fetus, pulsatile or continuous. Such a study has been done in the gonadotropin-releasing hormone pulse generator cells (*12*). However, the corticotropin cells are more randomly dispersed in the adenohypophysis, which would make such an *in vivo* experiment difficult to perform.

In a study measuring both cortisol and ACTH in fetal sheep every five minutes over a period of two hours, pulses (as analyzed by frequency domain methods) of ACTH occurred with a frequency of two per hour at both 133 and 143 dGA (*13*). The amplitude of the pulses (34 $pg.ml^{-1}$) was not different at the two ages, while the mean concentration of ACTH rose (*13*). Recently, Brooks and Challis (1991) have reported fetal ovine plasma ACTH pulses occurring on the average of one every 40 minutes in fetuses sampled for six hours at ten minute intervals at 140 dGA (*14*). They found much higher variability, but similar means, in their samples (*14*) than those seen in the current study. Both the rate and the amplitude of the pulses (*13, 14*) were within the range of detection of PULSAR and the time series. The difference in results may be attributable to the different pulse detection methods.

Myometrial contractures produced by the administration of oxytocin to the pregnant ewe result in the release of ACTH by the fetus (15,16). This stimulus to fetal ACTH secretion requires the fall in fetal PO_2 that generally accompanies a contracture (16). In several studies the frequency of contractures during late pregnancy in sheep has been shown

to vary from one every 30 to 60 minutes (17,18). Our study made no attempt to specifically sample fetal blood in relation to individual contractures.

Adult rats, sampled either for four hours at a rate of two minutes, or for six hours at a rate of fifteen minutes (*1*), indicated a much more complex pulse pattern than has been shown here. The spectral analysis performed indicated rhythms of plasma ACTH concentrations with periodicities between four and 220 minutes. This observation further supports the idea that the pulses detected in the ovine fetus were not for the maintenance of basal plasma ACTH levels.

CONCLUSION

The lack of significance in the age group comparisons suggests that during development of the hypothalamo-pituitary-adrenal cortical axis there are no changes in pulse amplitude or frequency of ACTH. These results do not support the idea that more than one sample needs to be taken to obtain a valid measurement of the unstressed baseline fetal plasma ACTH. However, responses to experimental stimulation may be pulsatile. ACTH concentrations during fetal hypoxia or hypotension should be investigated in a similar manner.

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Figure 1 Twelve-sample series of Fetal Plasma ACTH values from Fetus 3006 over the 125-129 dGA window at the four sampling rates.

1

Figure 1

Table 1 Individual Regression Lines of ACTH versus dGA.

Fetus	Slope	R^2	F-statistic
2057	1.02	31.7%	7.51*
2107	2.26	82.1%	51.52*
3001	2.98	65.7%	14.40*
3006	1.82	56.5%	20.49*

*significant at the 5% level.

Table 2 Mean plasma ACTH for Individual Time Series

Fetus	dGA Window	2 minute	5 minute	10 minute	30 minute
3006	**125**	20.43	19.98	19.15	18.30
	130	18.33	22.78	22.69	25.39
	135	46.52	47.56	56.65	54.04
	140	44.82	41.95	30.31	45.97
2107	**125**	--	--	--	--
	130	24.60	24.91	24.56	26.65
	135	30.74	33.46	34.29	30.25
	140	53.99	39.98	41.94	58.67
3001	**125**	--	--	--	--
	130	36.51	35.31	34.42	27.14
	135	50.36	46.22	40.21	46.17
	140	--	--	--	--
2057	**125**	22.77	12.68	16.31	20.37
	130	20.88	26.40	20.76	26.30
	135	15.03	10.37	23.55	19.47
	140	35.12	58.85	--	29.94

Table 3 Individual % Coefficients of Variation of ACTH

Sheep	dGA Window	2 minute	5 minute	10 minute	30 minute
3006	**125**	7.9	9.2	7.6	9.2
	130	14.4	4.4	9.3	67.9
	135	5.6	10.7	7.6	15.8
	140	8.1	9.5	21.1	28.7
2107	**125**	--	--	--	--
	130	9.8	4.3	21.0	40.9
	135	17.4	35.7	33.5	17.1
	140	7.3	12.0	26.1	28.5
3001	**125**	--	--	--	--
	130	35.2	16.4	18.8	15.5
	135	15.1	10.5	12.0	11.3
	140	--	--	--	--
2057	**125**	89.8	41.1	8.6	7.8
	130	6.3	9.2	6.1	12.2
	135	14.0	8.2	21.1	27.2
	140	15.4	6.8	--	19.8

Table 4 Description of 19 fetal plasma ACTH pulses organized by sampling rate: number of pulses per sample series, number of points composing a pulse, and pulse amplitude.

	2 min	5 min	10 min	30 min
1 pulse[a]	2	3	3	3
2 pulse[b]	0	0	1	3
2 points[c]	1	2	0	3
<1 SD[d]	0	1	0	1
>1 SD	1	1	2	6
>2 SD	0	0	3	1
>3 SD	1	1	0	1

[a]Number of 12-point sample series at a given sampling rate which contained one pulse.

[b]Number of 12-point sample series at a given sampling rate which contained two pulses.

[c]Number of 12-point sample series at a given sampling rate which contained a pulse composed of two points (all other pulses are composed of only one point).

[d]Pulse amplitude in units of standard deviation above mean of sample series (including pulse values).

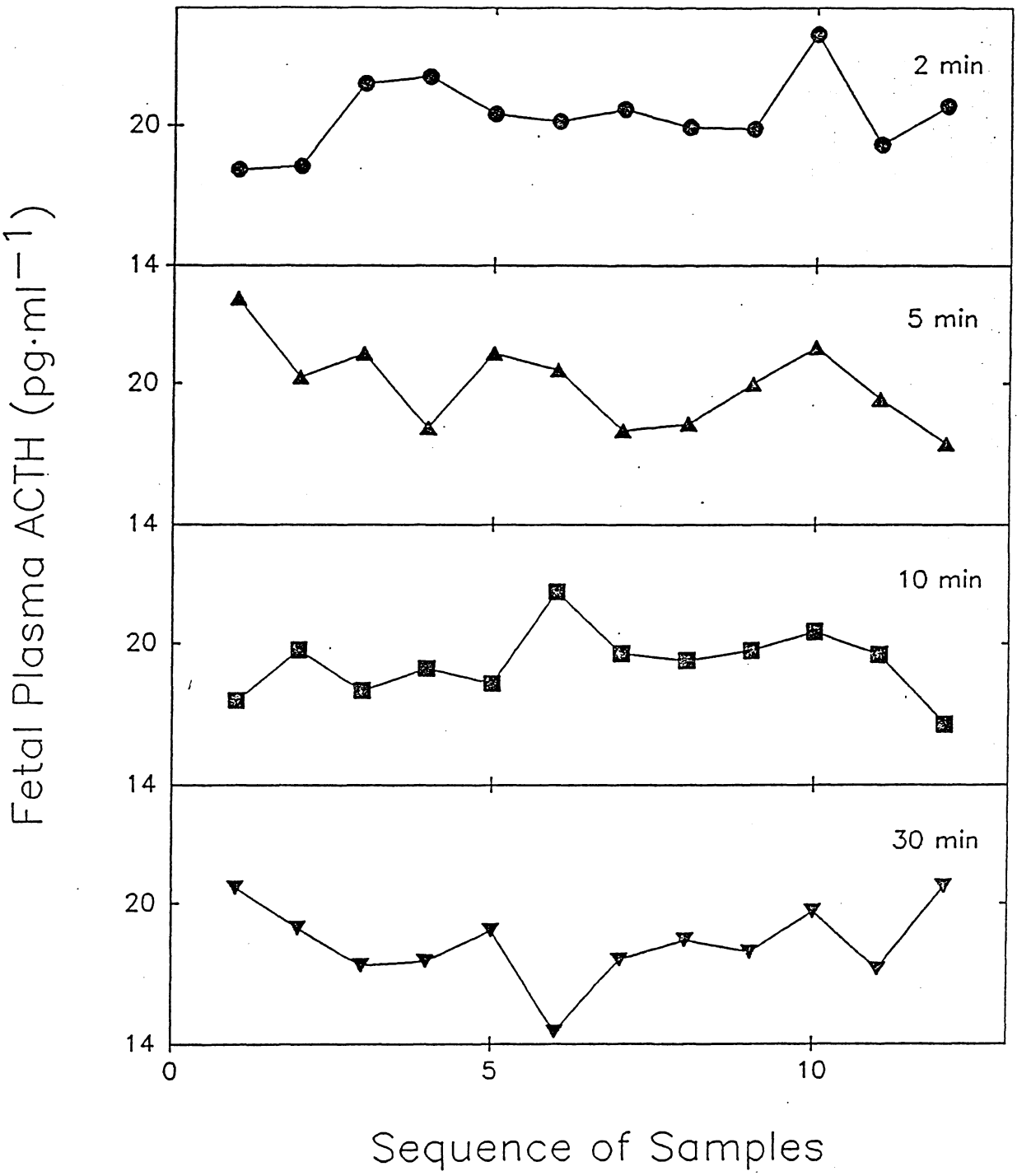
2 min
5 min
10 min
30 min
20
14
0
5
10
Fetal Plasma ACTH (pg·ml⁻¹)
Sequence of Samples

Figure 5.1