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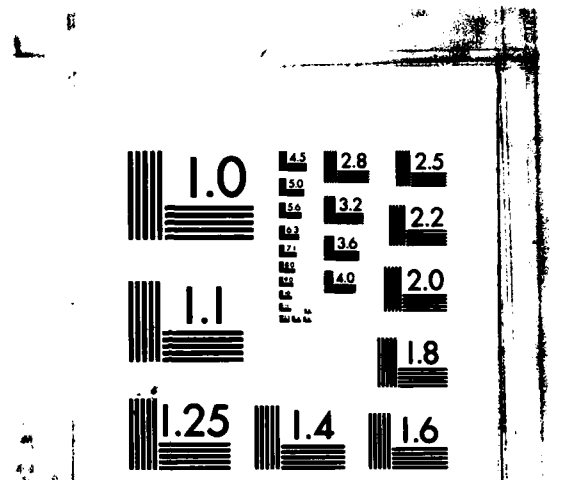

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USE OF SMALL ALIQUOTS OF FROZEN RED BLOOD CELLS AS CONTROLS FOR IN VITRO P_{50} MEASUREMENTS OVER EXTENDED PERIODS

by

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Small aliquots of red cells with low, normal and high P_{50} values were frozen with 40% W/V glycerol and stored at -80 C for at least 1 year. After thawing and washing, these red blood cells were used as controls for P_{50} measurements. In studies conducted over a 3-year period, this quality control system has been shown to be a convenient, stable and reproducible way to assess the instruments used for P_{50} measurements as well as the technicians conducting the tests.

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ABSTRACT

Small aliquots of red cells with low, normal and high P50 values were frozen with 40% W/V glycerol and stored at -80 C for at least 1 year. After thawing and washing, these red blood cells were used as controls for P_{50} measurements. In studies conducted over a 3-year period, this quality control system has been shown to be a convenient, stable and reproducible way to assess the instruments used for P_{50} measurements as well as the technicians conducting the tests.

INTRODUCTION

The P_{50} measurement, which defines the oxygen tension at which hemoglobin is 50% saturated with oxygen, has been in existence in one form or another for over 75 years. Although many of the currently used methods are precise, most are complex and require specialized equipment and considerable technical expertise utilizing a variety of sample preparation techniques and having little day-to-day quality control standards.

A recently licensed method of biochemically treating human red blood cells to increase their 2,3 DPG levels and thus their P50 values[1] requires quality control standards from the time of blood collection to the time of transfusion. What is needed in addition to the usual blood banking standards is a simple, reliable, reproducible, and quality controlled method of measuring red blood cell oxygen affinity, or P_{50}.

We have previously reported on a quality control system for P_{50} developed at the Naval Blood Research Laboratory.[2] This technique involves the cryopreservation of small aliquots of red blood cells, and we present here data collected over a 3-year period using this system to quality control methods of measuring red cell P_{50}. This same technique has been used to make P_{50} measurements in fresh blood, nonwashed and washed liquid-stored red cells, and previously frozen washed red cells.

METHODS

P_{50} Control Measurements

The normal P_{50} range was established from 4 units of ABO and Rh compatible packed red blood cells stored in citrate-phosphate-dextrose at 4 C for 0-2 days as follows: A 6.2 M solution of glycerol was added to each unit of red blood cells to achieve a final glycerol concentration of 40% W/V. The individual units of glycerolized red blood cells were pooled, mixed, and then divided into about 200 small aliquots. Each aliquot, containing a 10 ml volume of glycerolized red blood cells, was frozen in a 50 ml polypropylene sterile tube (Corning Glassworks, Corning, NY) at -80 C to serve as an example of a normal P_{50}.

Small aliquots of red blood cells with low P_{50} values were prepared from 4 units of compatible packed red blood cells stored at 4 C for 14 to 21 days as follows: each unit was glycerolized to a 40% W/V concentration, pooled, mixed, and divided into aliquots, and frozen at -80 C as described above.

The high P_{50} red blood cells were prepared from 4 units of compatible packed red blood cells stored at 4 C for 6-7 days as follows: A 50 ml volume of PIPA Solution C, containing 100 mM/l pyruvate, 100 mM/l inosine, 100 mM/l phosphate, and 5 mM/l adenine, pH 7.2, osmolality 500 mOsm/kg H_2O, was added to each unit of red blood cells and the unit was incubated for 1 hour at 37 C to increase the red cell 2,3 DPG level. Each unit of biochemically modified red cells with a high P_{50} value was glycerolized to a concentration of 40% W/V, pooled, mixed, divided into aliquots and frozen

at -80 C as described above.

On each day over a 3-year period, at least 2 units of frozen red blood cells with different P_{50} values were thawed by placing them in a 37 C water bath for precisely 10 minutes. Each thawed red cell sample was washed as follows: A 2.5 ml volume of 12% NaCl was added to the thawed aliquot in the original freezing tube with gentle mixing. After 2 minutes of equilibration, a 10 ml volume of 0.9% NaCl solution containing 0.2% glucose and 25 mEq/l disodium phosphate, pH 6.8, was added slowly with gentle mixing, followed by 2 minutes of equilibration. The red blood cells were centrifuged at 2400 rpm at room temperature for 2.5 minutes, and then washed 3 times with 10 ml of 0.9% NaCl-0.2% glucose-25 mEq/l disodium phosphate, pH 6.8. The P_{50} of each sample of red blood cells was measured in duplicate after calibration of the P_{50} analyzer.

Measurement of P_{50}

The Hemoscan Dissociation Analyzer (American Instruments Co., Silver Spring, MD) was used to make P_{50} measurements. This instrument combines a 2-wavelength spectrophotometer, a Clark oxygen electrode, and an X-Y plotter to display the entire oxygen dissociation curve.[2,3] Each red blood cell sample was washed 4 times in phosphate-buffered saline (PBS) containing 0.9% NaCl and 0.10% disodium phosphate, osmolality 300 mOsm/kg H_2O, buffered to pH 7.8 with 1.0 N NaOH. After the final wash, the red blood cells were resuspended to a hematocrit value of approximately 50% in PBS solution.

A single drop of the sample was placed on a glass coverslip, covered

with a membrane, and placed in a holder in the sample compartment for 15 minutes to allow the temperature of the sample to equilibrate to a temperature of 37 C. A tank of 25% oxygen and 75% nitrogen and a tank of 100% nitrogen were used, and these gases were mixed and delivered to the sample chamber automatically to increase PO_2 from 0-180 torr. The outputs of the spectrophotometer and oxygen electrode were simultaneously displayed on the Y and X axis of the plotter respectively. The P_{50}, without correction for pH, was read directly from the plot. Since a very thin red blood cell suspension allows rapid oxygen diffusion into the red blood cells, a hematocrit value of 40-60% in the red cell suspension was necessary to be within the spectrophotometer sensitivity to record percentage of oxygen saturation accurately. The PBS solution did not have the buffering capacity to adjust the red blood cells with hematocrits of 40-60% to uniform pH values. After final resuspension, the red cell suspension pH was measured at 37 C in an IL 813 Blood Gas Analyzer (Instrumentation Laboratories, Lexington, MA) and a pH correction was made. The linear pH correction factor used was: corrected P_{50} = measured P_{50} - 29.3 (7.2-pH), the derivation of which was reported previously.[2] In this system, P_{50} of the washed red blood cells in PBS solution was measured at 37 C, at a 0 torr PCO_2, and corrected to a pH of 7.2. The oxygen electrode and spectrophotometer were calibrated with each sample at 0 and 100% saturation and the entire oxygen dissociation curve was plotted. The sample chamber allowed for "conditioning" of one sample while another was being scanned. Five of 7 samples can be measured in 1 hour, including washing, sample preparation, and pH correction.

Daily Measurement of Controls

On a daily basis over a 3-year period, at least 2 aliquots of red blood cells with different P_{50} values were thawed, washed, and measured. A total of 18 laboratory technicians were involved in the preparation and evaluation of these samples throughout this period. Duplicate measurements of pH and P_{50} were made on each sample. The lot number of each control level, the duplicate P_{50} and pH measurements, the duplicate pH correction P_{50} values, and the technician number were recorded.

The data were entered into an HP-9845 computer and analyzed using a HP statistical analysis package (Hewlett-Packard, Lexington, MA).

RESULTS

Results of the first 50 days of the study using small aliquots of frozen red blood cells are reported elsewhere.[2] During the 3 years of study reported here, there were 5 lots of red blood cells with high P_{50} values, 5 with normal P_{50} values, and 3 with low P_{50} values. Most of the 18 technicians were medical technology students with less than 1 year of laboratory experience. Table 1 shows the lot number for the aliquot of red cells, the mean P_{50} value obtained on the first measurement of the day, the number of days that samples of red cells from a specific lot were run, and the standard deviation of the P_{50} measurement for each lot. The data show that the P_{50} values in the aliquots of red cells frozen by this technique were consistent for all groups (Table 1). Figure 1 shows the first of two P_{50} measurements on each red cell aliquot shown as mm Hg, and the mean $\pm$ 2 standard deviations. Variations were smallest in the first lots studied. Larger variations were seen in lots used during the second year, and the variations decreased during the third year. The increase in variations observed in lots used in the second year may have been due to the utilization of newly trained technicians, use of a second Hemoscan Analyzer, and electrical power surges in the laboratory which caused malfunctions in many instruments and affected the operation of the Hemoscan.

The values obtained by individual technicians for each lot of red cell samples with high, normal, and low P_{50} values were analyzed to determine if any individual routinely obtained values higher or lower than the mean value;

no discrepancy was found. The red cells with high P_{50} values had a correlation coefficient of 0.65 between the first and second daily values; the red cells with normal P_{50} values had a correlation coefficient of 0.80; and the red cells with low P_{50} values had a correlation coefficient of 0.77 between the two values. The mean values and standard deviations of the first of duplicate measurements were in all cases similar to the mean values of the second measurement. The combined correlation coefficient between the first and second of duplicate measurements for all lots was 0.73 ± 0.17.

There was no consistent increase or decrease in P_{50} values that correlated with the number of days the controls from each lot were measured. There was no significant change in the variation between duplicate samples during the use of each lot of high, normal, and low P_{50} red cell controls, indicating that each lot of small aliquot frozen red cells maintained a stable P_{50} value during the period it was used (Table 1).

DISCUSSION

With the recent licensure of biochemically modified red blood cells for routine clinical transfusion, measurement of P_{50} is indicated to cwntrol the quality of the preserved red cells. In the past, a sample of freshly collected blood was used to determine the accuracy of the instrument to measure P_{50} values. The red cell 2,3 DPG and P_{50} values deteriorate rapidly during liquid storage at 4 C. By freezing small aliquots of low, normal, and high P_{50} red cells, long-term P_{50} stability was achieved. Our lots of frozen red cells remained stable for the 12 months they were in use, and small aliquots were used to train technicians. Before testing unknown blood samples, each of the 18 technicians had to demonstrate the ability to thaw, deglycerolize, and wash red cells with low, normal, and high P_{50} values, to calibrate the Hemoscan and Blood Gas Analyzer, to perform the testing of pH and P_{50}, and to obtain a pH corrected P_{50} value within a $\pm$ 2 S.D. of the mean of each lot. Following deglycerolization, controls are prepared and measured like samples of fresh, liquid-stored or previously frozen red cells. In order for red cells with different P_{50} values to be within range, the Hemoscan, pH correction, technique, temperature and buffer composition must all be correct. The control P_{50} values were out of range for the following reasons: a defective thermostat, hyperosmolar buffer, the wrong oxygen composition in the calibrating gas tank, a second sample slide jammed in the sample chamber of the Hemoscan,and several electronic malfunctions in the Hemoscan which allowed normal calibration but faulty operation.

The small aliquot glycerol-frozen red blood cells with high, normal, and low P_{50} values used for quality control can only be frozen at -80 C; they do not remain stable at -20 C. Equipment needed includes a -80 C freezer, and a 37 C water bath and centrifuge for thawing and washing. Deviations from the thawing procedure as outlined were found to alter the P_{50} values. The procedure requires a total of about 45 minutes to thaw and wash 2 or 3 red cell samples, 20 minutes to calibrate the Blood Gas Analyzer and Hemoscan, and 1-1/4 hours to run the 3 red cell samples in duplicate. During most of this time constant attention is not required. Additional P_{50} samples can be run at the rate of 5 to 7 per hour. Time can be saved if red cell samples are not run in duplicate.

For over 3 years we have been freezing small aliquots of red blood cells with high, normal and low 2,3 DPG levels for use as a quality control of the instrumentation for P_{50} measurements as well as the personnel conducting these tests. We have found this procedure to be convenient, stable, and reproducible.

FIGURE 1

The initial control values of the low, normal, and high P_{50} red blood cell controls on a daily basis over a period of 1073 days are shown. The means $\pm$ 2 S.D. for each lot are also included.

TABLE 1

SMALL ALIQUOT FROZEN RED BLOOD CELL P_{50} CONTROL VALUES

Lot Number	Time of Testing	Length of Storage of RBC at 4 C Prior to Freezing	Mean	n	Standard Deviation
High P_{50} Range					
3	2/7/79-2/20/80	7 Day Old Treated with Sol. C	41.09	160	1.01
5	2/27/80-6/24/80	7 Day Old Treated with Sol. C	40.58	64	1.24
7	6/25/80-1/29/81	7 Day Old Treated with Sol. C	42.36	130	1.42
10	1/27/81-6/12/81	7 Day Old Treated with Sol. C	42.04	82	1.55
12	6/10/81-12/23/81	7 Day Old Treated with Sol. C	42.12	126	1.39
Mean	2/7/79-12/23/81	7 Day Old Treated with Sol. C	41.69		1.45
Normal P_{50} Range					
1	1/17/79-8/30/79	1 Day Old Non Treated	29.54	112	1.17
4	7/16/19-8/14/80	2 Day Old Non Treated	29.62	197	1.56
8	8/15/80-9/24/80	1 Day Old Non Treated	29.64	27	2.12
9	10/24/80-6/18/81	2 Day Old Non Treated	28.26	134	1.88
13	6/22/81-12/23/81	o Day Old Non Treated	30.17	117	1.54
Mean	1/17/79-12/23/81	1.2 Day Old Non Treated	29.41		1.74
Low P_{50} Range					
2	1/24/79-3/14/80	14 Day Old Non Treated	17.76	174	.95
6	3/11/80-1/26/81	22 Day Old Non Treated	15.84	107	1.32
11	2/4/81-9/11/81	21 Day Old Non Treated	17.74	10	1.63
Mean	1/24/79-9/11/81	19 Day Old Non Treated	17.05		1.45

TABLE 2A

SMALL ALIQUOT FROZEN RBC P_{50} CONTROLS. A COMPARISON OF THE VALUES OBTAINED BY SEVERAL DIFFERENT TECHNICIANS

High P_{50} Range

Mean Lot Values				Individual Technician Values			
Lot #	Mean	n	S.D.	Technician#	Mean	n	S.D.
3	41.09	160	1.01				
				1	41.36	71	.78
				2	40.22	13	.73
				3	40.45	10	1.12
				4	40.5	12	1.14
				5	41.13	26	.89
				8	41.27	24	1.31
				9	41.38	4	1.14
5	40.58	64	1.24				
				3	40.70	2	.85
				4	39.90	18	1.30
				5	41.17	7	1.39
				8	40.67	6	.74
				9	40.54	5	1.20
				10	40.92	15	.58
				11	40.86	5	1.91
				12	40.10	3	.37
				13	40.93	3	.93
7	42.36	130	1.42				
				3	40.2	1	---
				4	42.89	33	1.50
				5	41.72	34	1.09
				8	40.40	44	1.20
				10	42.46	15	1.92
				11	43.2	2	1.27
				12	44.2	1	---
10	42.04	82	1.55				
				4	41.95	4	1.50
				5	42.10	26	1.75
				10	42.10	24	1.43
				14	41.93	28	1.54
12	42.12	126	1.39				
				4	41.09	8	1.96
				5	41.90	22	1.28
				14	42.46	7	1.42
				16	42.26	71	1.43
				17	41.72	15	.83
				18	43.1	3	.86

TABLE 2B

SMALL ALIQUOT FROZEN RBC P_{50} CONTROLS. A COMPARISON OF THE VALUES OBTAINED BY SEVERAL DIFFERENT TECHNICIANS

Normal P_{50} Range							
	Mean Lot Values			Individual Technician Values			
Lot #	Mean	n	S.D.	Technician#	Mean	n	S.D.
1	29.54	112	1.17				
				1	29.73	78	1.22
				2	29.29	25	.75
				3	29.10	6	1.31
				4	27.95	2	.78
				6	27.2	1	---
4	29.62	197	1.56				
				1	29.56	8	1.33
				2	28.92	14	1.03
				3	28.57	6	1.78
				4	29.14	45	1.87
				5	30.06	51	1.48
				6	27.00	1	---
				8	30.01	34	1.17
				9	28.52	10	1.69
				10	30.47	17	1.14
				11	29.76	5	1.36
				12	29.73	3	1.31
				13	29.83	3	.72
9	28.26	134	1.88				
				4	29.59	14	1.71
				5	27.70	34	2.05
				8	28.28	18	1.98
				10	28.60	32	1.38
				12	29.27	3	2.45
				14	27.79	33	1.84
13	30.17	117	1.54				
				4	31.20	7	1.49
				5	29.53	20	1.36
				14	28.75	2	.64
				16	30.44	70	1.48
				17	29.71	15	1.19
				18	28.87	3	3.27

TABLE 2C

SMALL ALIQUOT FROZEN RBC P_{50} CONTROLS. A COMPARISON OF THE VALUES OBTAINED BY SEVERAL DIFFERENT TECHNICIANS

Low P_{50} Range

Mean Lot Values				Individual Technician Values			
Lot #	Mean	n	S.D.	Technician#	Mean	n	S.D.
2	17.76	174	.95				
				1	17.64	73	.81
				2	16.89	24	.95
				3	17.37	6	.54
				4	17.75	13	1.14
				5	18.47	19	---
				6	16.85	2	1.20
				8	18.40	29	.71
				9	17.97	8	.51
6	15.84	107	1.32				
				3	15.05	2	1.34
				4	15.82	26	1.34
				5	15.35	12	1.75
				8	16.14	34	.92
				9	14.30	1	---
				10	15.69	23	1.48
				11	16.20	6	1.87
				12	16.40	2	.28
				13	15.40	1	---

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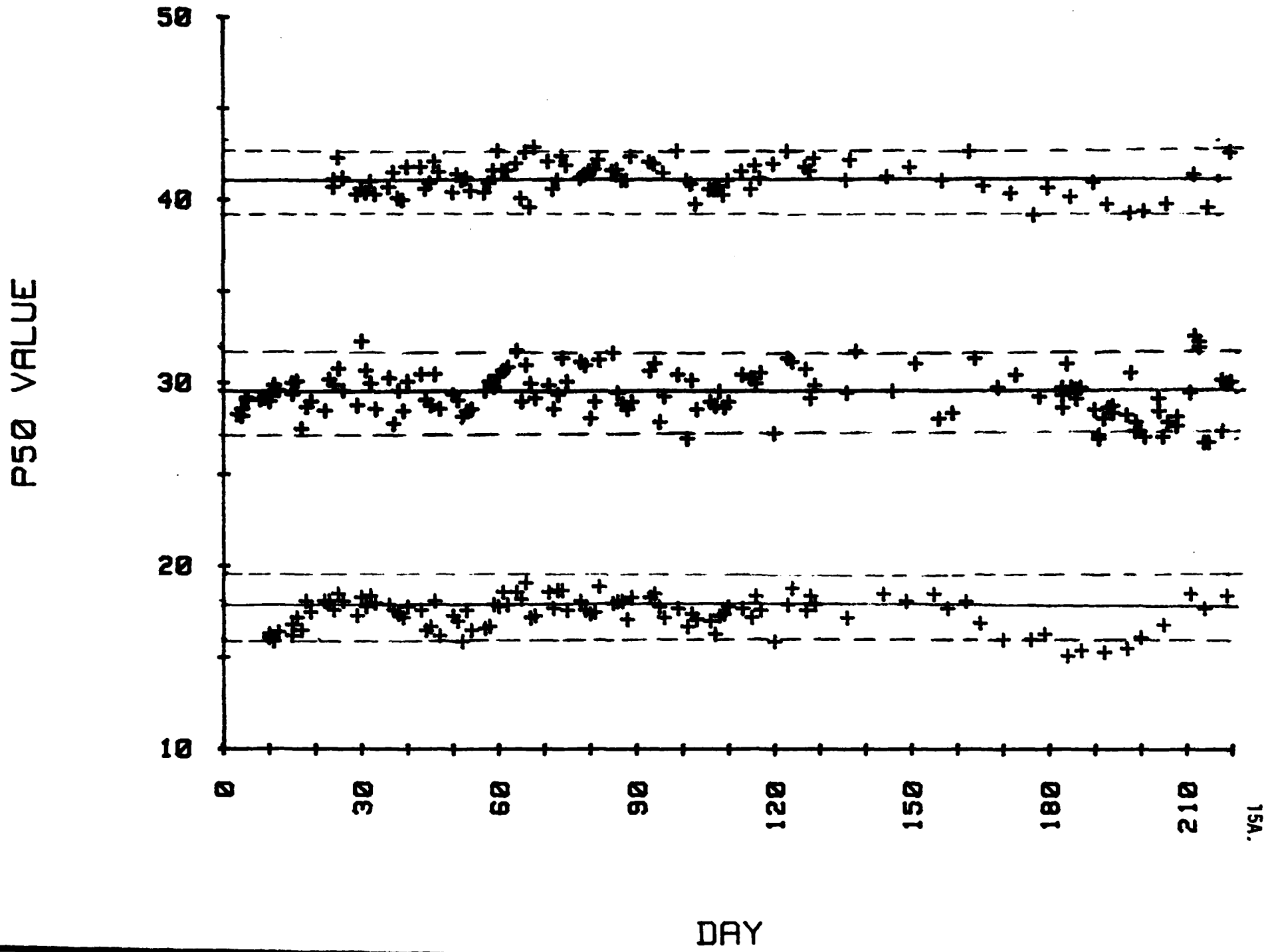
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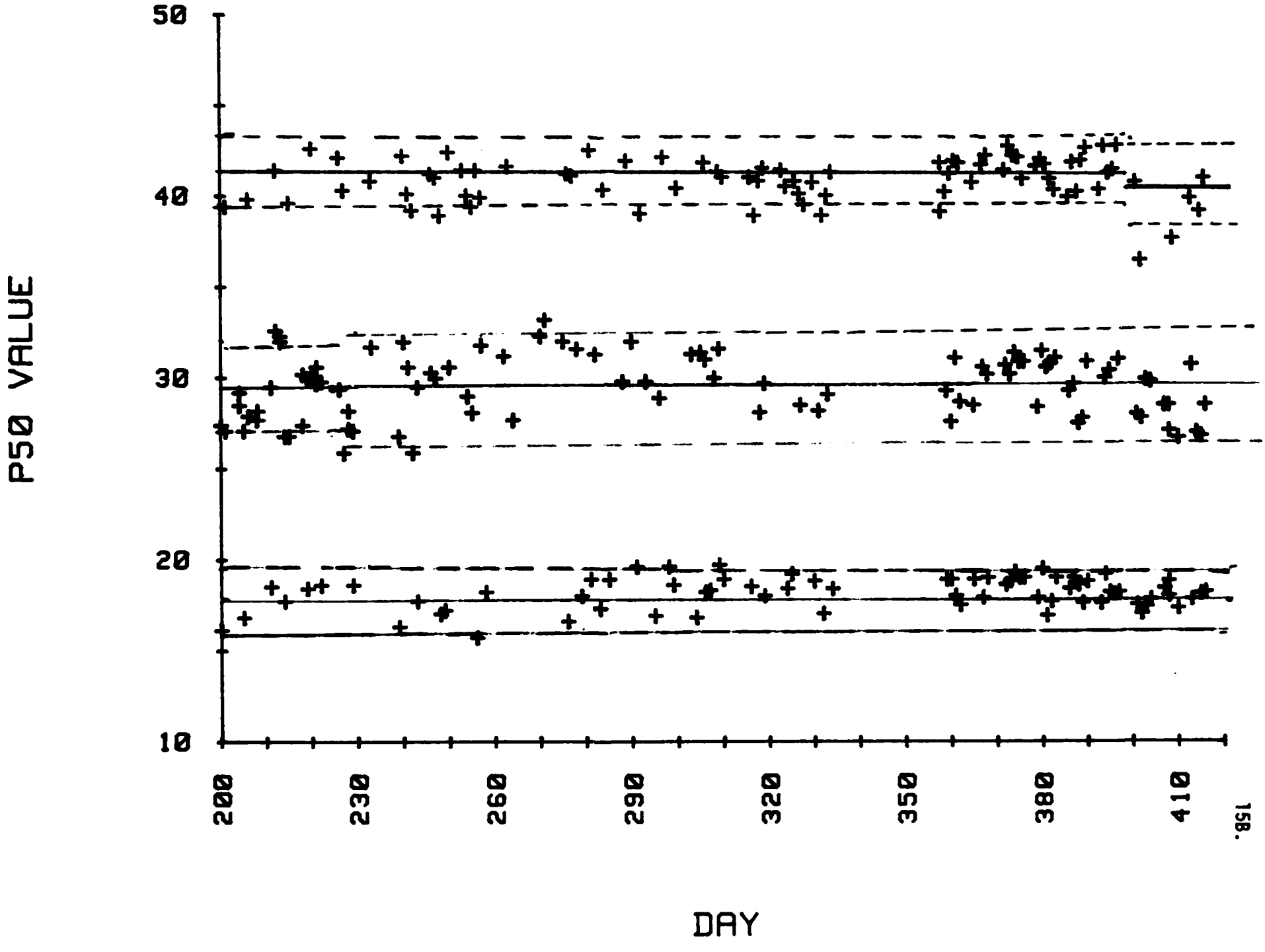
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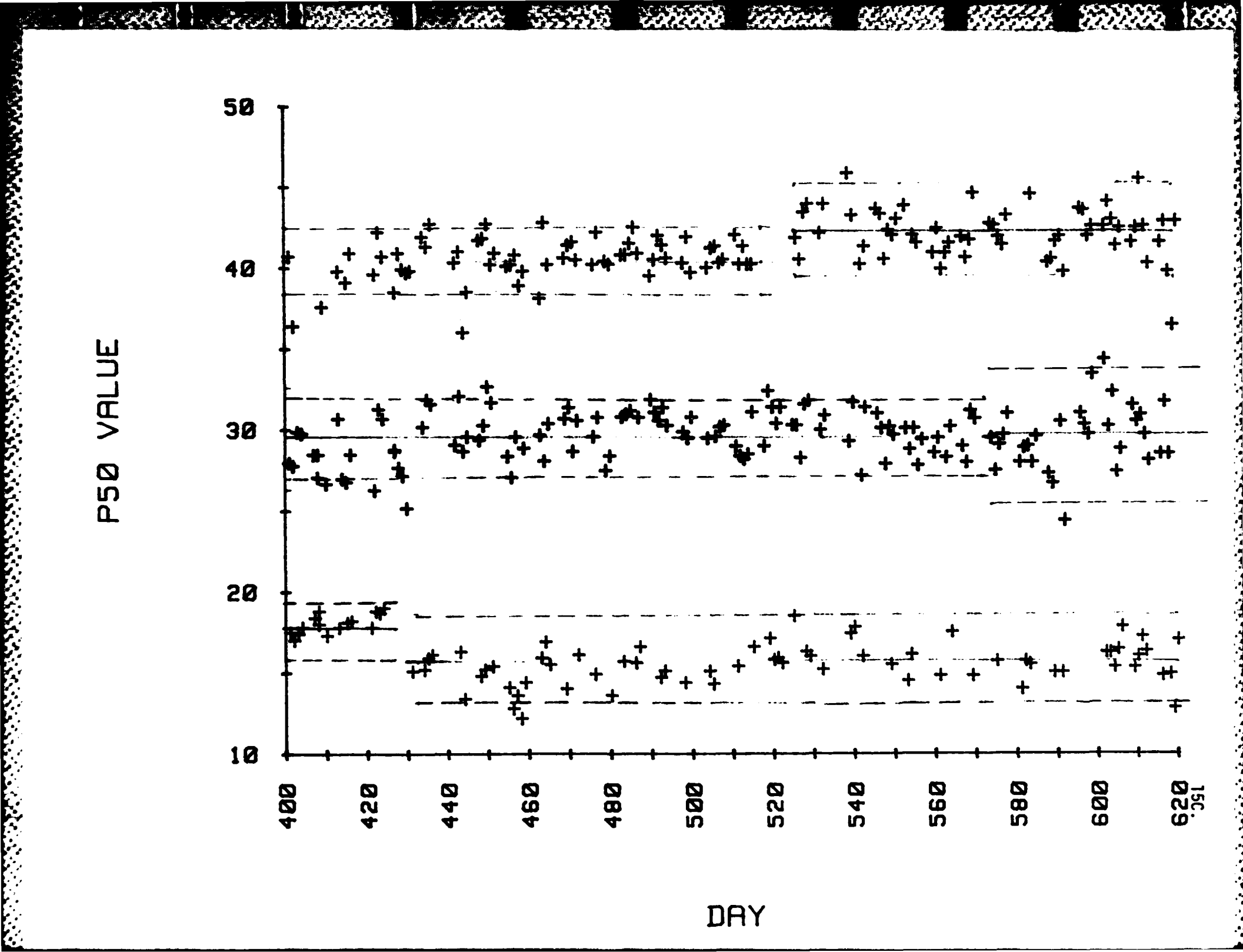
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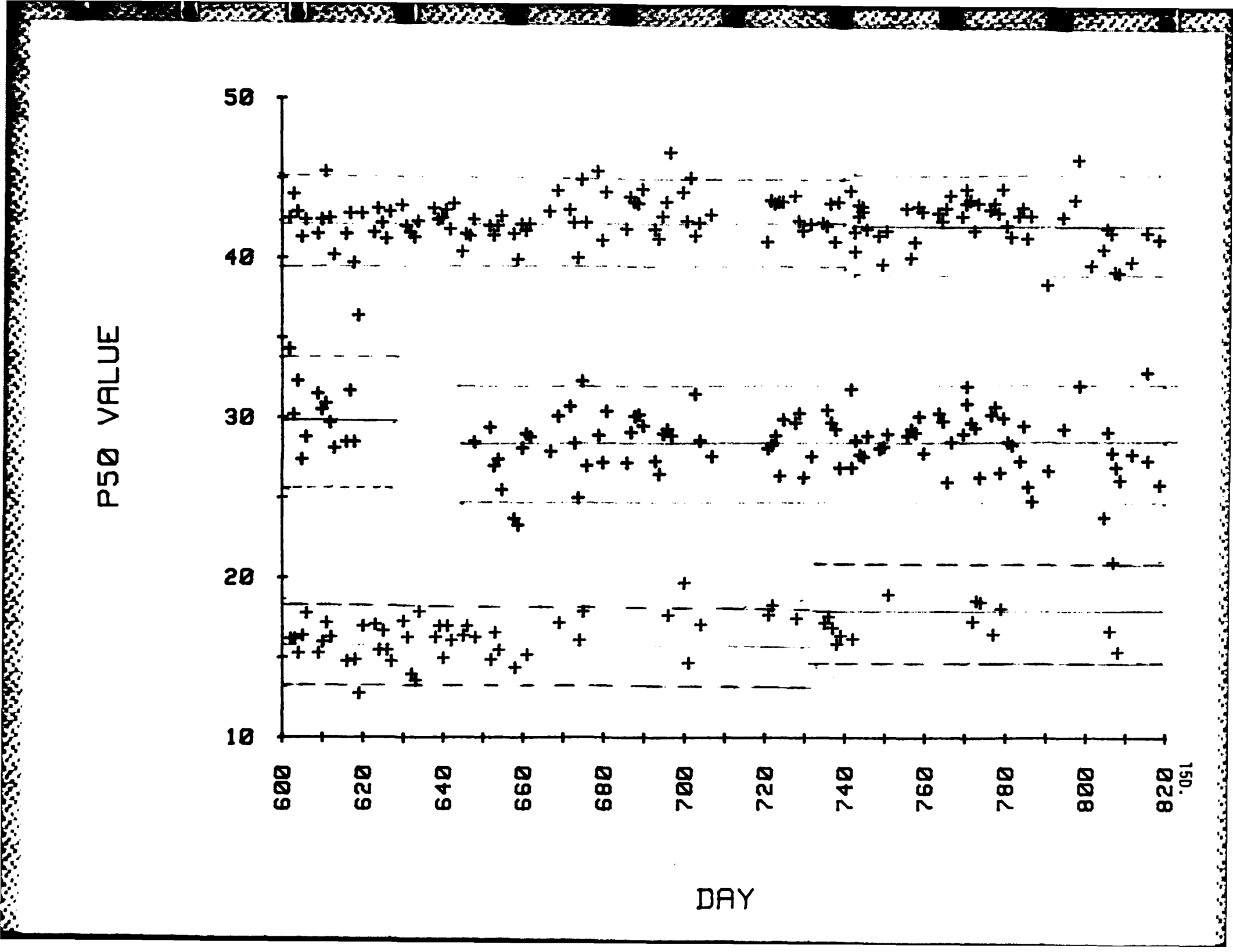
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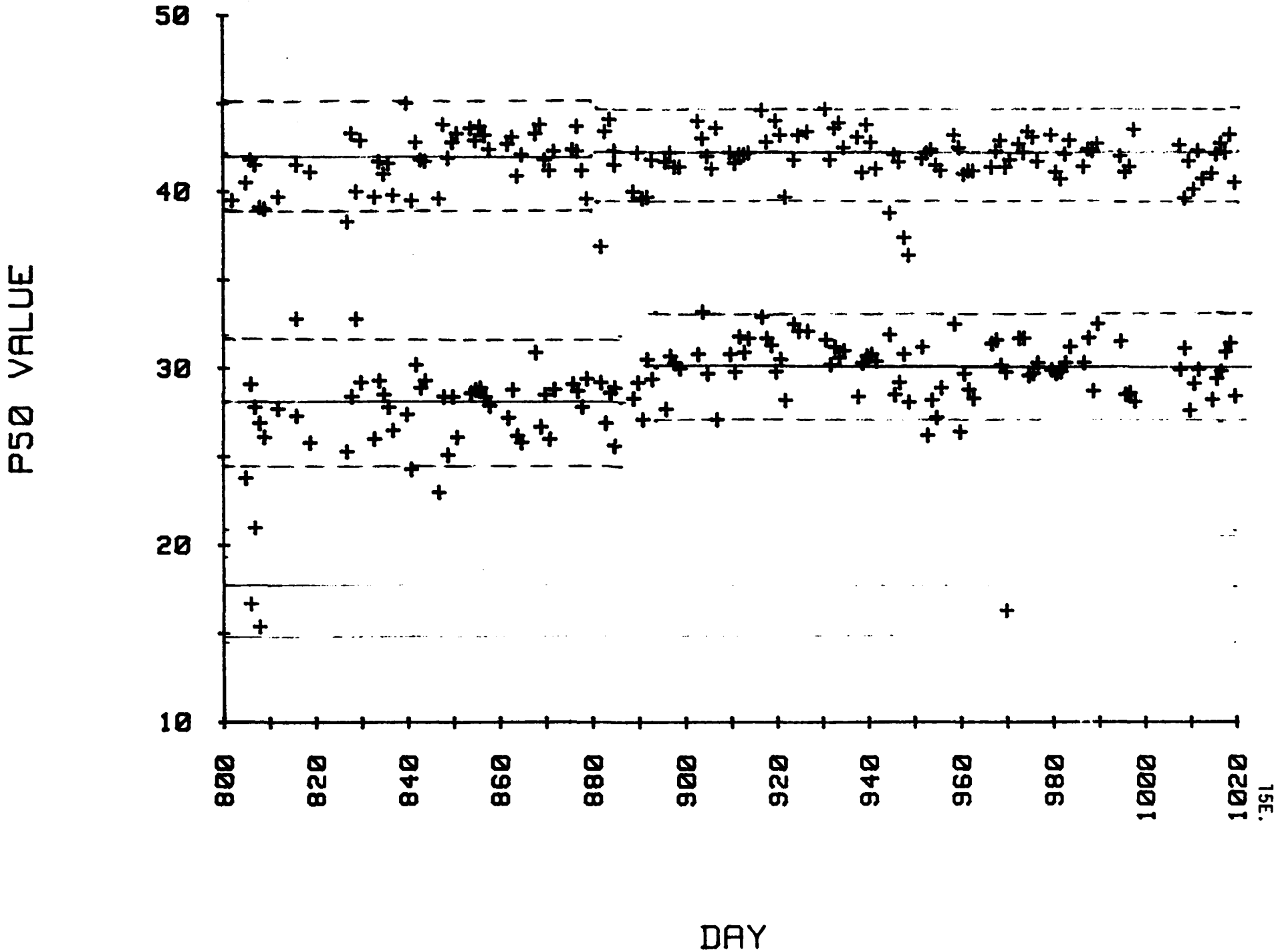
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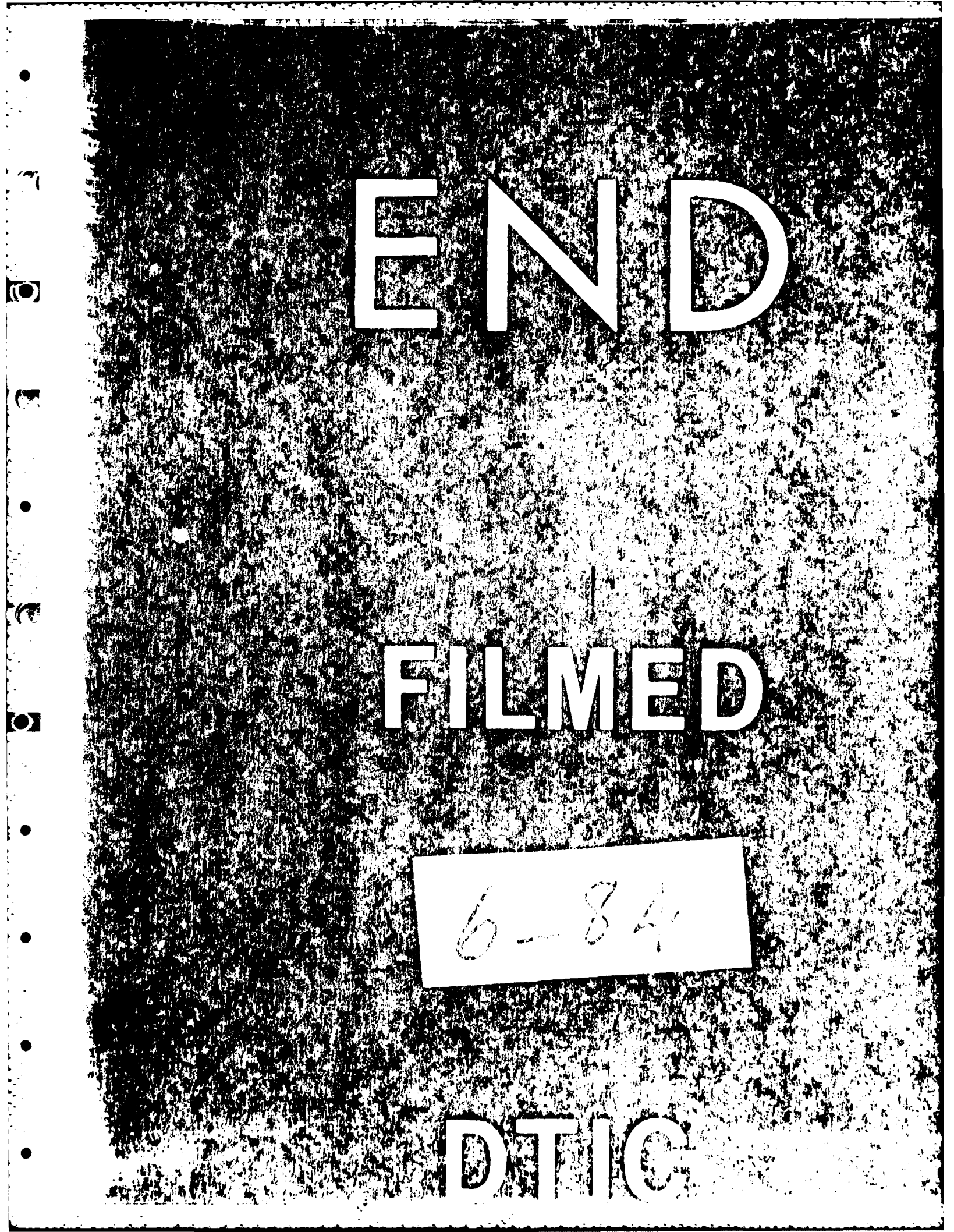
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