A COMPARATIVE SURVEY OF IMMUNOGLOBULIN A AND THIOCYANATE LEVELS IN SALIVA

by

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CHAPTER 1

INTRODUCTION

In vivo, Immunoglobulin A (IgA) recognizes foreign antigens and binds with them to aid the phagocytosis of foreign material by macrophagens. It can also trigger a more widespread reaction of the immune system with T-cell and B-cell involvement (Barnett, 1978; Lehner et al., 1974). Evidence indicates that IgA may inhibit the binding of potentially pathogenic micro-organisms of the mucous membranes and thus prevent infection (Weir, 1977). IgA is also thought to bind food particles that may be potential allergens (Weir, 1977). As one of the primary immunoglobulins, IgA is important in both acquired and innate immunity as a protective mechanism (Barnett, 1978; Rose et al., 1979).

In adults, serum IgA is found in levels ranging from 0.2-0.3gm/100ml, or about 2.4-3.9% of the total serum proteins, while IgA of saliva ranges from .020-.20gm/100ml (Brown et al., 1975). Synthesized at approximately 1.7gm/day for 70kg body weight, IgA has a half-life of six days. Similar to IgG, IgA has four peptide chains, two light and two heavy, in a ratio of 6 IgG: 1 IgA (Weir, 1977). Serum IgA occurs as a monomer (mol.wt. 155,000) and

sedimentation coefficient of 7S, while the dimeric, trimeric, and tetrameric forms have sedimentation coefficients of 10-17S (Rose et al., 1979; Sack et al., 1980).

In the monomeric form, IgA is bivalent and can be subclassified into two forms, IgA1 and IgA2 which occur in a ratio of 85 IgA1 to 15 IgA2 (Rose et al., 1979). IgA2 is unusual in that the light chains in this subclass are linked to each other by disulfide bonds, rather than the heavy chains bonding in this manner (Underdown et al., 1977).

IgA is found primarily in the external secretions such as colostrum, tears, nasal and respiratory mucous, intestinal mucous, and saliva (Cunningham, 1978). IgA2 is found in the total makeup of the secretions (60%) such as saliva, whereas IgA1 is the primary constituent of total serum IgA (90%) (Arglebe, 1981).

Thiocyanate (SCN^-) is a natural byproduct of cyanide degradation in the body and is found in small quantities in saliva, gastric juices, and other body secretions (Boxer and Rickards, 1952; Neurath, 1972). It can be acquired by consumption of apricots, almonds, radishes, eggs, turnips, and cassava root (Bible and Chong, 1975; Bourdous et al., 1978; Sapeika, 1974; Montgomery and Lierner, 1969). Its natural presence in the saliva makes thiocyanate a desirable comparison compound to IgA due to the fact that no extraneous chemicals need be introduced to the sample.

Research of the biochemical role of sulfur in thiocyanate has well established its capacity in the

detoxification of cyanide (Boxer and Rickards, 1952; Wood et al., 1947).

The influence of thiocyanate in essential hypertension has been demonstrated (Astwood, 1943; Rawson et al., 1943) to be related to the breakdown of sodium nitroprusside into cyanide by reacting with tissue and blood sulfhydryl groups. Following this, it is converted from cyanide to thiocyanate via an enzyme, rhodanese (sulfur transferase), and the presence of sodium thiosulfate (Saunders and Himwich, 1950). Furthermore, Boxer and Rickards (1952), employing C^{14}-labeled cyanide and thiocyanate, postulated that the in vivo equilibrium between the two must be far in the direction of thiocyanate in view of the observed 1:1000 ratio of cyanide to thiocyanate in body fluids. Recent studies have shown potassium thiocyanate (KSCN), the form utilized in this analysis, inhibits activities of trypsin and chymotrypsin on casein by mixed competitive and noncompetitive inhibition (Ivankovich et al., 1978; Shikimi et al., 1979). This suggests that potassium thiocyanate may play more than a passive role in physiological alteration of biological enzymes.

This study compared variations in saliva IgA to changes in saliva thiocyanate. The quantitative precipitin assays of IgA in saliva samples of diverse levels of thiocyanate were analyzed statistically to determine if a significant correlation existed between the two.

Alteration of secreted byproducts involved in

metabolism (ex. thiocyanate) and any interaction or alteration of biological protective components (ex. Immunoglobulin A) would accentuate current understanding of fluctuations in innate immunity or other protective mechanisms.

CHAPTER II

EXPERIMENTAL PROCEDURE

Materials

Potassium thiocyanate, ferric nitrate, sodium perchlorate, phosphorus pentoxide, and Brij-35 were secured through Fisher Scientific, Fair Lawn, New Jersey. Spectrum^{R} filters (10,000μ pore size) and ultrafiltration unit were purchased from Medical Industries, Los Angeles. The RID (radial immunodiffusion) secretory and nonsecretory anti-IgA sera, diffusion plates, and reference sera were purchased from ICL Scientific, Fountain Valley, California.

Saliva samples were obtained from the Texas Research Institute of Mental Sciences, Houston, Texas, and University of Houston (central campus) by permission.

Methods

Preparation of Saliva Samples

Saliva samples that were to be used for thiocyanate and IgA assay were collected by placing a dental roll in the mouth until saturated. The saliva-wetted dental roll was placed in a tightly capped 12 × 75mm plastic test tube and preserved at -5°C prior to analysis.

Ultrafiltration of the Saliva Samples

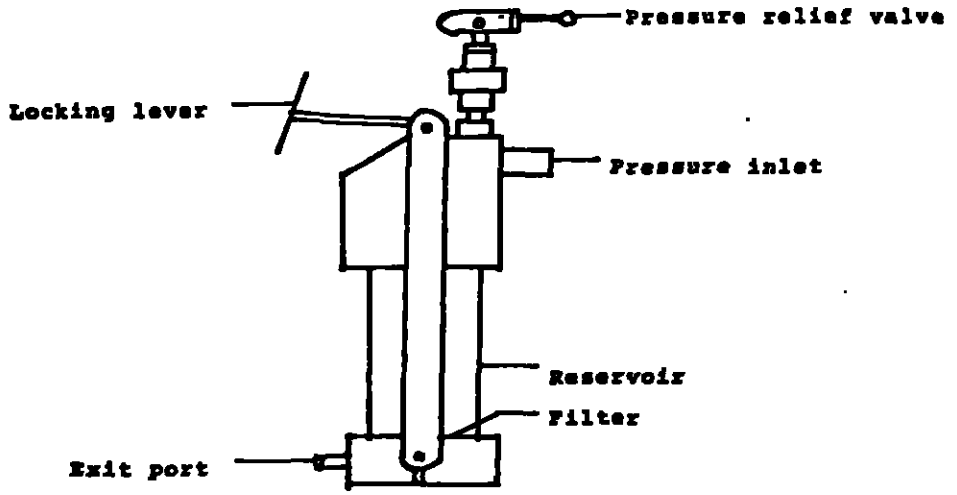
Samples selected for analysis were washed with 3 ml deionized water in a Spectrum^{R} (Medical Industries, Inc., 60916 Terminal Annex, Los Angeles 90054) pressure filtering system of pore size 10,000 microns and 50 psig to remove extraneous materials including salts. This apparatus is illustrated in figure 1.

The filtrate was tested using the gel diffusion method to verify that the IgA did not pass through the filter.

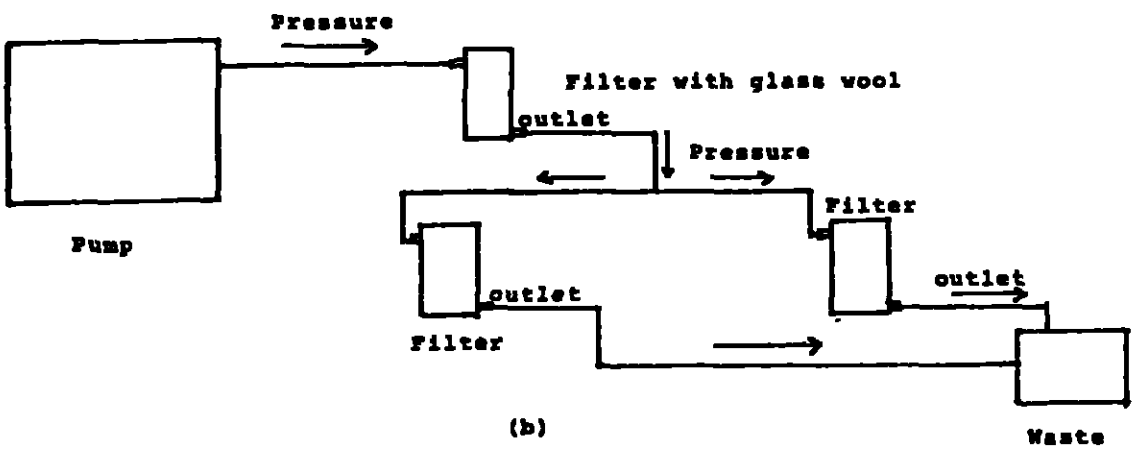
The samples were reconstituted with 0.3-0.7mls of water following pressure application and washing. Forceful pipetting with the water loosened any material adhering to the filter surface. The reconstituted filtrate was suctioned off and placed in the original tube for concentration.

Lyophilization of the Saliva Samples

The saliva samples were concentrated via a lyophilization apparatus described in figure 2. A dozen samples at a time were placed in a large desiccator jar, which in turn was attached to a cold trap via a sleeve connection. The cold trap, utilizing liquid nitrogen, offers a large surface area. This apparatus was connected to a Welsh Duo-seal 1/3 hp vacuum pump which allowed the concentration of the samples in a vacuum. The samples to be analyzed were frozen at a slant to avoid loss due to the vacuum.



(a)



(b)

Figure 1. (a) Spectra por Filter^{R} (Ultrafiltration discs, MWCO: 10,000, pH range: 1-14, disc Diameter: 43mm., H_2O flowrate 0.4 ml/min/cm^2 @4 kg/cm^2.)
(b) A schematic diagram of the Immunoglobin A filtering process.

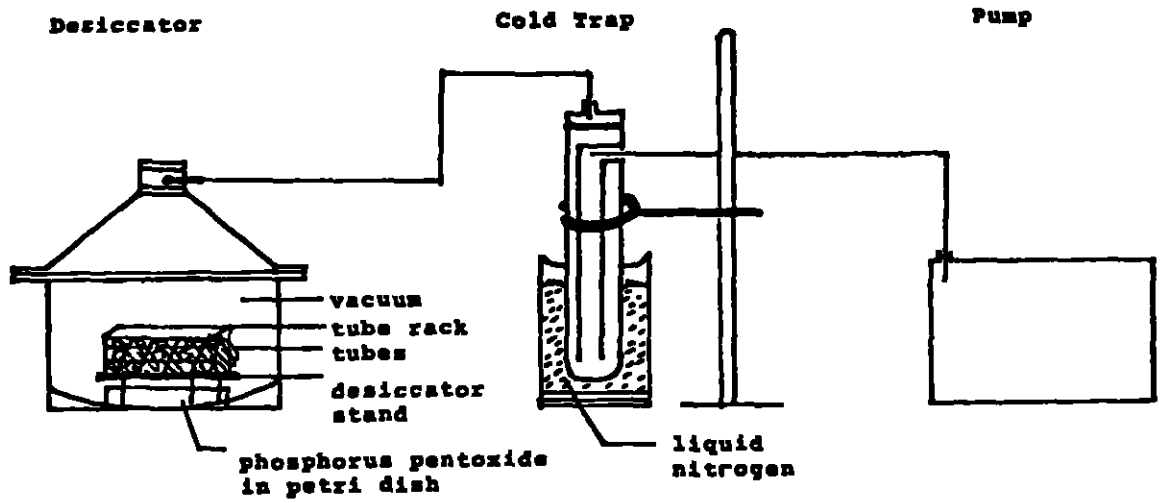


Figure 2. Apparatus for the lyophilization of Immunoglobin A.

Phosphorus pentoxide, located beneath the tubes, was employed to absorb any excess water from the samples. For dry storage, the tubes were placed in a large desicator jar containing calcium chloride.

Concentration of Saliva Samples

Final concentration was obtained by adding distilled water to the lyophilized samples until the desired concentration was achieved. Previous experiments demonstrated that 5-10X concentrations of the original amount was an effective range of concentration.

Immunoglobulin Quantification by Radial Immunodiffusion

The quantification of IgA was established by utilizing standardized regular and ultralow level anti-sera gel diffusion plates.

Quantification of IgA was according to the methods described by Milford-Ward (1977) and demonstrated in figure 3. Assayed sera containing 53mg%, 162mg%, and 480mg% IgA were added to wells in agarose containing anti-IgA. The diameter of the precipitin ring around the standard sample well was measured via a Kallestad Calibrating RID Viewer and compared to the wells to which saliva was added to determine the specific amount of IgA present.

Total Protein Determination

Total protein determinations of the concentrated samples were obtained via a modified Lowry (1951) procedure

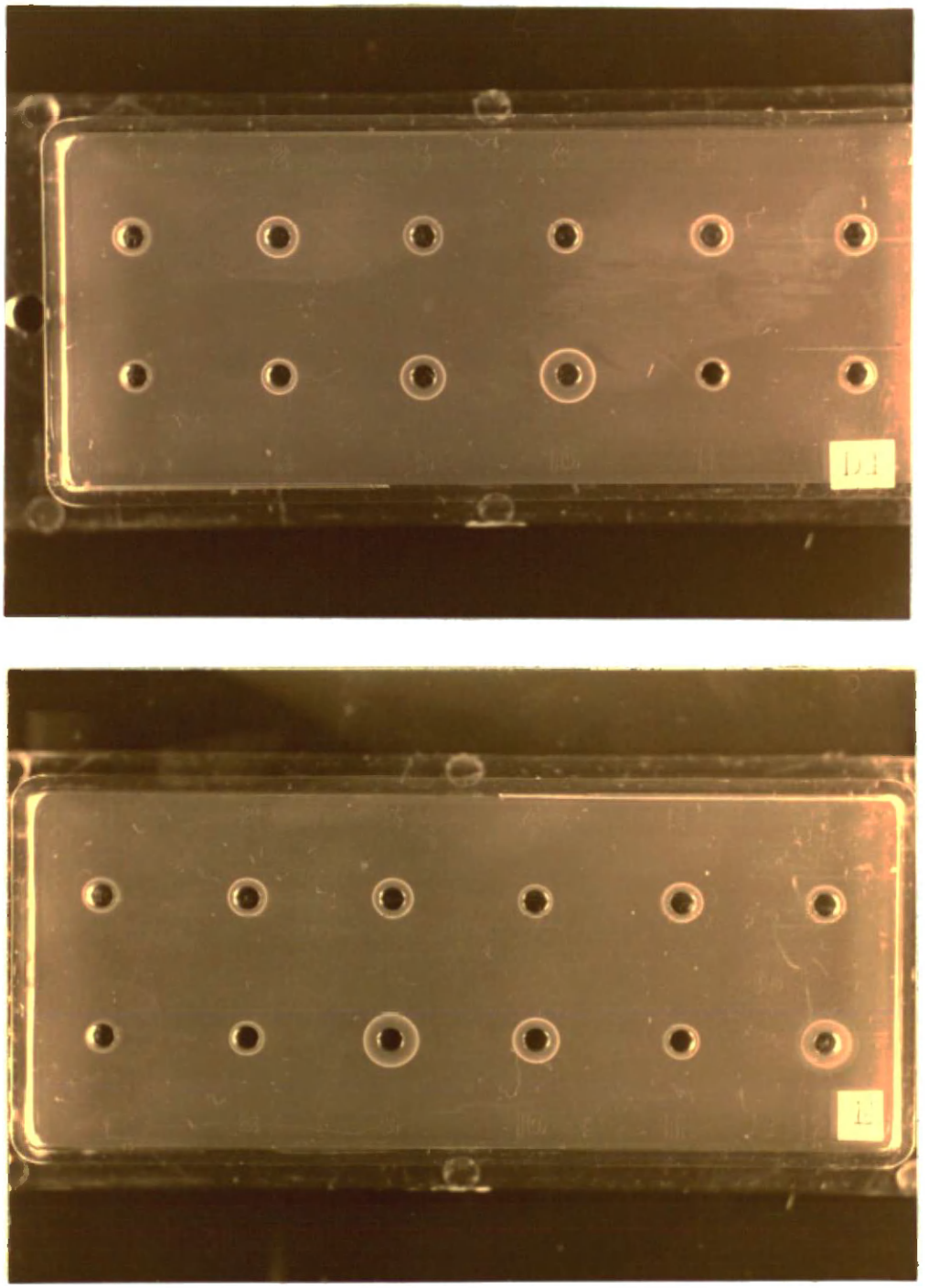


Fig. 3. Radial immunodiffusion of saliva IgA. Plates D_1 and E demonstrate the antisera-IgA precipitin reaction of experimental samples, while wells E9-11 contain stds. of 480mg%, 162mg%, and 53mg%.

using the Folin Ciocalteu (1927) reagent and reduced µl volumes to accommodate the low sample volumes. Bovine serum albumin was used for the protein standard and spectrophotometric analysis (750 n.m.) was performed on a Beckman DB-GT grating spectrophotometer.

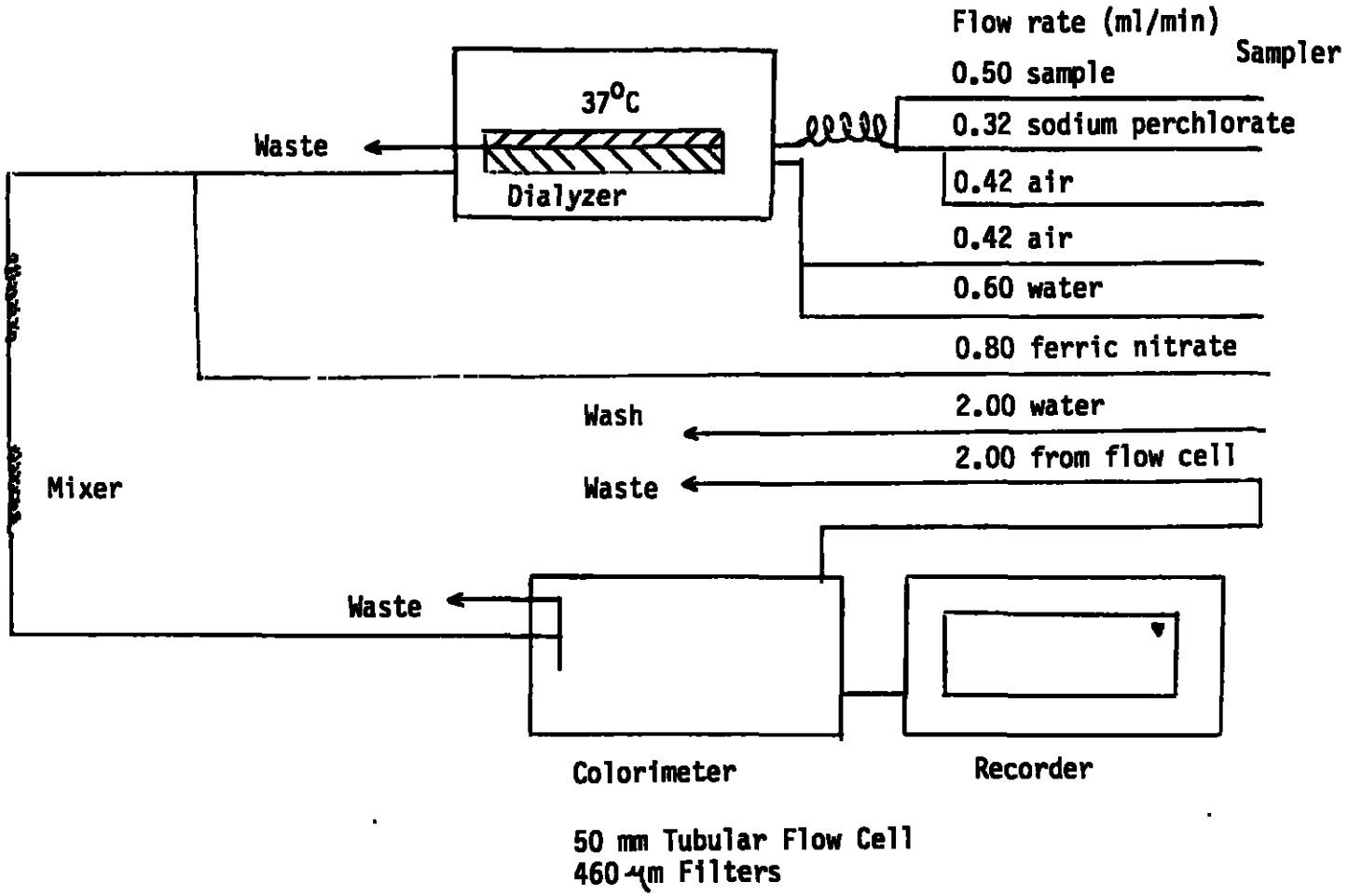
Thiocyanate Analysis of Saliva Samples

To extract the saliva for thiocyanate anlaysis, the dental roll was thawed and placed in a 20cc syringe and the sample expressed into a 2.0ml plastic autoanalyzer sampling cup. The average sample size of 0.6ml. The Technicon autoanlayzer was used to analyze the saliva samples for thiocyanate (Butts et al., 1974). The flow diagram for the autoanalyzer is shown in figure 4 (Giraudia and Grillo, 1981).

To obtain the mg/ml readings, ferric nitrate (10.0gm/l ferric nitrate in 1.5M nitric acid solution) was used to give a color reaction which absorbed at 460nm. The thiocyanate was separated from the sample by a reaction with sodium perchlorate [12.5gm/l sodium perchlorate in 1ml Brij-35 (30gm/dl) and a wash solution of 1ml Brij-35/1l of water]. Standards of 20, 50, 100, 150, and 200 µg/ml of potassium thiocyanate were used to establish a standard curve. Saliva samples containing 0-150 µg/ml and 150-300 µg/ml of thiocyanate were used for the IgA determination.

Upon obtaining this data, the proposed comparison between IgA and thiocyanate levels could be made. Statistical analyses, such as determination of central

Figure 4. Flow diagram for thiocyanate determination with the Technicon AutoAnalyzer I.



tendency, polynomial regression, and skewness were applied to determine if any significant correlation of difference existed between the two. The distribution of the sample readings for IgA with regard to the 0-150 µg/ml and 150-300 µg/ml groups of thiocyanate were also examined with these methods as well as frequency distribution histograms, to illustrate statistical parameters.

Concentration of the samples, original sample volume, and degree of reaction in the gel diffusion were all variables to be considered.

CHAPTER III

RESULTS

Quantitation of Immunoglobulin A and Thiocyanate in Human Saliva

Upon observation of the radial immunodiffusion reaction if IgA and anti-IgA sera, a range of values (5-510mg/100ml) was noted.

The total protein levels from the Lowry (1951) method of protein estimation were between 5-102.5mg/ml. Table I represents the IgA and protein levels obtained.

The automated colorimetric analysis of thiocyanate, which provided values from 29-294mg/ml can also be observed in table I.

The mathematical analysis of IgA and thiocyanate are presented in figures 5 through 7. A frequency histogram of the data is given to demonstrate the skewed nature of each distribution. The statistical parameters utilized are delineated in tables II and II. The division of values into respective intervals and cumulative subtotals, as well as descriptive statistics of central tendency (mean, median, and mode), skewness, kurtosis (peakness), and standard deviation are also provided.

Thiocyanate values are presented in table IV with

Table I: Experimental values determined for Immunoglobulin A (mg/dl), thiocyanate (mg/ml), and total protein (mg/ml).

IgA		Thiocyanate	Protein
trial 1	trial 2		
26	24	47	31
26	21	29	20
5	7	56	8
7	13	56	17
10	13	68	30
14	26	58	35
52	40	92	7
8	13	92	37
11	11	46	23
159	90	66	78
138	113	114	57
113	26	68	31
13	16	130	23
5	4	76	14
38	44	80	56
17	18	122	31
68	90	72	32
24	24	80	40
30	42	97	61
14	10	78	22
138	113	52	25
333	306	56	67
200	200	38	66
421	465	130	102
30	40	108	15
90	138	60	27
54	54	118	20
17	17	69	33
280	427	203	33
230	345	234	62
30	50	191	99
201	250	203	73
370	427	294	41
215	82	294	14
26	4	248	22
350	11	190	71
102	175	286	14
157	223	184	31
53	17	156	7
30	90	262	18
10	198	156	26
135	175	160	12
40	50	230	11
67	115	262	39
101	268	262	29
410	510	208	95
10	54	218	5
61	140	262	24
10	30	230	17
67	82	256	20
102	115	216	34
5	50	242	18
109	82	270	12
67	63	156	6
381	63	230	11
10	11	294	5

HISTOGRAM OF VARIABLE 1 RUN 1

Symbol X Count 56 Mean 101.827 STd. Dev. 116.811

Interval Name	. 5 10 15 20 25 30 35 40	Frequency Int.	Frequency Cum.	Percentage Int.	Percentage Cum.
40.000	XXXXXXXXXXXXXXXXXXXXXXXXXXXX	26	26	46.4	46.4
80.000	XXXXXXXXXX	8	34	14.3	60.7
120.00	XXXXXXXX	6	40	10.7	71.4
160.00	XXXXXXX	5	45	8.9	80.4
200.00	X	1	46	1.8	82.1
240.00	XXX	3	49	5.4	87.5
280.00	X	1	50	1.8	89.3
320.00		0	50	0.0	89.3
360.00	XX	2	52	3.6	92.9
400.00	XX	2	54	3.6	96.4
LAST	XX	2	56	3.6	100.0
	5 10 15 20 25 30 35 40				

Figure 5. Frequency Distribution of Immunoglobulin A (Trial 1).

HISTOGRAM OF VARIABLE 2 RUN 2

Symbol X Count 56 Mean 101.827 Std. Dev. 116.811

Interval Name	5 10 15 20 25 30 35 40	Frequency Int.	Frequency Cum.	Percentage Int.	Percentage Cum.
40.000	XXXXXXXXXXXXXXXXXXXXXXXX	21	21	37.5	37.5
80.000	XXXXXXXXXXX	9	30	16.1	53.6
120.00	XXXXXXXXXXXX	10	40	17.9	71.4
160.00	XX	2	42	3.6	75.0
200.00	XXXXXX	5	47	8.9	83.9
240.00	X	1	48	1.8	85.7
280.00	XX	2	50	3.6	89.3
320.00	X	1	51	1.8	91.1
360.00	X	1	52	1.8	92.9
400.00		0	52	0.0	92.9
LAST	XXXX	4	56	7.1	100.0
	5 10 15 20 25 30 35 40				

Figure 6. Frequency Distribution of Immunoglobulin A (Trial 2).

HISTOGRAM OF VARIABLE 3 THIO

Symbol X Count 56 Mean 152.768 Std Dev. 84.543

Interval Name	5 10 15 20 25 30 35 40	Frequency Int:	Cum.	Percentage Int.	Cum.
30.000	X	1	1	1.8	1.8
60.000	XXXXXXXXX	9	10	16.1	17.9
90.000	XXXXXXXXX	9	19	16.1	33.9
120.000	XXXXXX	6	25	10.7	44.6
150.000	XXX	3	28	5.4	50.0
180.000	XXXX	4	32	7.1	57.1
210.000	XXXXXX	6	38	10.7	67.9
240.000	XXXXXX	6	44	10.7	78.6
270.000	XXXXXXXX	8	52	14.3	92.9
LAST	XXXX	4	56	7.1	100.0
	5 10 15 20 25 30 35 40				

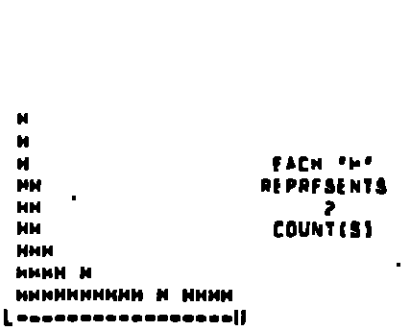
Figure 7. Frequency Distribution of Total Thiocyanate.

Table II Statistical distribution analysis for Immunoglobulin A (trial 1).

VARIABLE NUMBER	1	MAXIMUM	420.0000000
NUMBER OF DISTINCT VALUES .	21	MINIMUM	10.0000000
NUMBER OF VALUES COUNTED. .	56	RANGE	410.0000000
NUMBER OF VALUES NOT COUNTED	0	VARIANCE	13581.7471098
***VALUES ARE ROUNDED TO. .	10.0000	ST.DEV.	116.5409241
		(Q3-Q1)/2	62.5000000

LOCATION ESTIMATES

		ST.ERROR
MEAN	102.3214264	15.5736358
MEDIAN	50.0000000	17.3205166
MODE	10.0000000	



	VALUE	VALUE/S.E.
SKEWNESS	1.48	4.28
KURTOSIS	0.85	1.29

VALUE	COUNT	PERCENTS CELL	CUM	VALUE	COUNT	PERCENTS CELL	CUM	VALUE	COUNT	PERCENTS CELL	CUM	VALUE	COUNT	PERCENTS CELL	CUM
10.	14	25.0	25.0	90.	1	1.8	62.5	220.	1	1.8	85.7	380.	1	1.8	96.4
20.	3	5.4	30.4	100.	3	5.4	67.9	230.	1	1.8	87.5	410.	1	1.8	98.2
30.	7	12.5	42.9	110.	2	3.6	71.4	280.	1	1.8	89.3	420.	1	1.8	100.0
40.	2	3.6	46.4	140.	3	5.4	76.8	330.	1	1.8	91.1				
50.	3	5.4	51.8	160.	2	3.6	80.4	350.	1	1.8	92.9				
70.	5	8.9	60.7	200.	2	3.6	83.9	370.	1	1.8	94.6				

Table III. Statistical distribution analysis for Immunoglobulin A (trial 2).

VARIABLE NUMBER	2	MAXIMUM	510.0000000
NUMBER OF DISTINCT VALUES .	22	MINIMUM	0.0000000
NUMBER OF VALUES COUNTED. .	56	RANGE	510.0000000
NUMBER OF VALUES NOT COUNTED	0	VARIANCE	16373.7343750
***VALUES ARE ROUNDED TO. .	10.0000	ST.DEV.	127.9598923
		(Q3-Q1)/2	70.0000000

LOCATION ESTIMATES

		ST.ERROR
MEAN	111.6071396	17.0993595
MEDIAN	55.0000000	14.4337635
MODE	10.0000000	

```
H
H
H
H                         EACH "H"
HH                        REPRESENTS
HH                            2
HH                        COUNT(S)
HHH
HHHH H
HHHHHHHHH HH  HHH
L----------------U
```

EACH '-' ABOVE = 30.0000
L= 0.0000
U= 510.0000

	VALUE	VALUE/S.E.		
			Q1=	20.0000000
			Q3=	160.0000000
SKEWNESS	1.58	4.82	S-=	-16.3527527
KURTOSIS	1.68	2.57	S+=	239.5670319

EACH '.' BELOW = 5.0000

```
S    Q               Q       S
- M M 1    M     M   3       +                                  M
..I.O.......E..........E.......................................A
  N O       D          A                                        X
    E       I          N
```

VALUE	COUNT	PERCENTS CELL	PERCENTS CUM	VALUE	COUNT	PERCENTS CELL	PERCENTS CUM	VALUE	COUNT	PERCENTS CELL	PERCENTS CUM	VALUE	COUNT	PERCENTS CELL	PERCENTS CUM
0.	2	3.6	3.6	60.	2	3.6	53.6	180.	3	5.4	80.4	350.	1	1.8	92.9
10.	8	14.3	17.9	80.	3	5.4	58.9	200.	2	3.6	83.9	430.	2	3.6	96.4
20.	6	10.7	28.6	90.	3	5.4	64.3	220.	1	1.8	85.7	470.	1	1.8	98.2
30.	3	5.4	33.9	110.	2	3.6	67.9	250.	1	1.8	87.5	510.	1	1.8	100.0
40.	4	7.1	41.1	120.	2	3.6	71.4	270.	1	1.8	89.3				
50.	5	8.9	50.0	140.	2	3.6	75.0	310.	1	1.8	91.1				

Table IV. Statistical distribution analysis for thiocyanate.

VARIABLE NUMBER	1	MAXIMUM	290.0000000
NUMBER OF DISTINCT VALUES .	23	MINIMUM	30.0000000
NUMBER OF VALUES COUNTED. .	56	RANGE	260.0000000
NUMBER OF VALUES NOT COUNTED	0	VARIANCE	6971.2993164
***VALUES ARE ROUNDED TO. .	10.0000	ST.DEV.	83.4943085
		(83-61)/2	40.0000000

LOCATION ESTIMATES

		ST.ERROR
MEAN	153.2142792	11.1573963
MEDIAN	145.0000000	25.9807758
MODE	NOT UNIQUE	

EACH 'M' REPRESENTS 1 COUNT(S)

EACH '=' ABOVE = 20.0000
L= 0.0000
U= 340.0000

	VALUE	VALUE/S.E.
SKEWNESS	-0.20	-0.60
KURTOSIS	-1.49	-2.28

Q1= 70.0000000
Q3= 210.0000000
S= 69.7196707
S²= 2386.7065876

EACH '.' AFLOW = 2.0000

VALUE	COUNT	PERCENTS CELL	PERCENTS CUM	VALUE	COUNT	PERCENTS CELL	PERCENTS CUM	VALUE	COUNT	PERCENTS CELL	PERCENTS CUM	VALUE	COUNT	PERCENTS CELL	PERCENTS CUM
30.	1	1.8	1.8	90.	2	3.6	37.5	180.	1	1.8	58.9	240.	1	1.8	80.4
40.	1	1.8	3.6	100.	1	1.8	39.3	190.	2	3.6	62.5	250.	1	1.8	82.1
50.	3	5.4	8.9	110.	2	3.6	42.9	200.	2	3.6	66.1	260.	5	8.9	91.1
60.	5	8.9	17.9	120.	2	3.6	46.4	210.	1	1.8	67.9	270.	1	1.8	92.9
70.	5	8.9	26.8	130.	2	3.6	50.0	220.	2	3.6	71.4	290.	4	7.1	100.0
80.	4	7.1	33.9	160.	4	7.1	57.1	230.	4	7.1	78.6				

comparable statistical analysis.

A frequency distribution demonstrating the skewed nature of IgA (trials 1,2) appears in figures 5 and 6. Additionally, an analysis of individual frequencies related to the cumulative total are included.

The frequency analysis for thiocyanate, represented in figure 7, demonstates a more normalized frequency distribution than that of IgA.

In order to obtain overall insight to the nature of the comparison between IgA (trials 1,2) and thiocyanate, scatter diagrams of the individual values observed are presented in figures 8 and 9. These figures include mean, standard deviation, and regression coefficient values for reference.

Analysis of the relationship between the two trials of IgA reveals a correlation coefficient (r) of .754 indicating an overlap in variance of 57% (r^2 = .57). This correlation is raised to .900 (r^2) of 81% overlap by visual determination of outliers (those points found to be significantly removed from the principal information plotted). The effect of the removal of the two outliers determined is illustrated in figure 10.

As observed in figures 4 and 5, the IgA values (trials 1,2) exhibited a skewed distribution. The low linear regression coefficients, .1960 (y = .3221x + 52.617, trial 1) and .3105 (y = .4368x + 44.886, trial 2), are produced as a result of reduced correlation between IgA and thiocyanate due to skewness. Several transformations

Figure 8. Scatterdiagram of Immunoglobulin A (trial 1) plotted against thiocyanate.

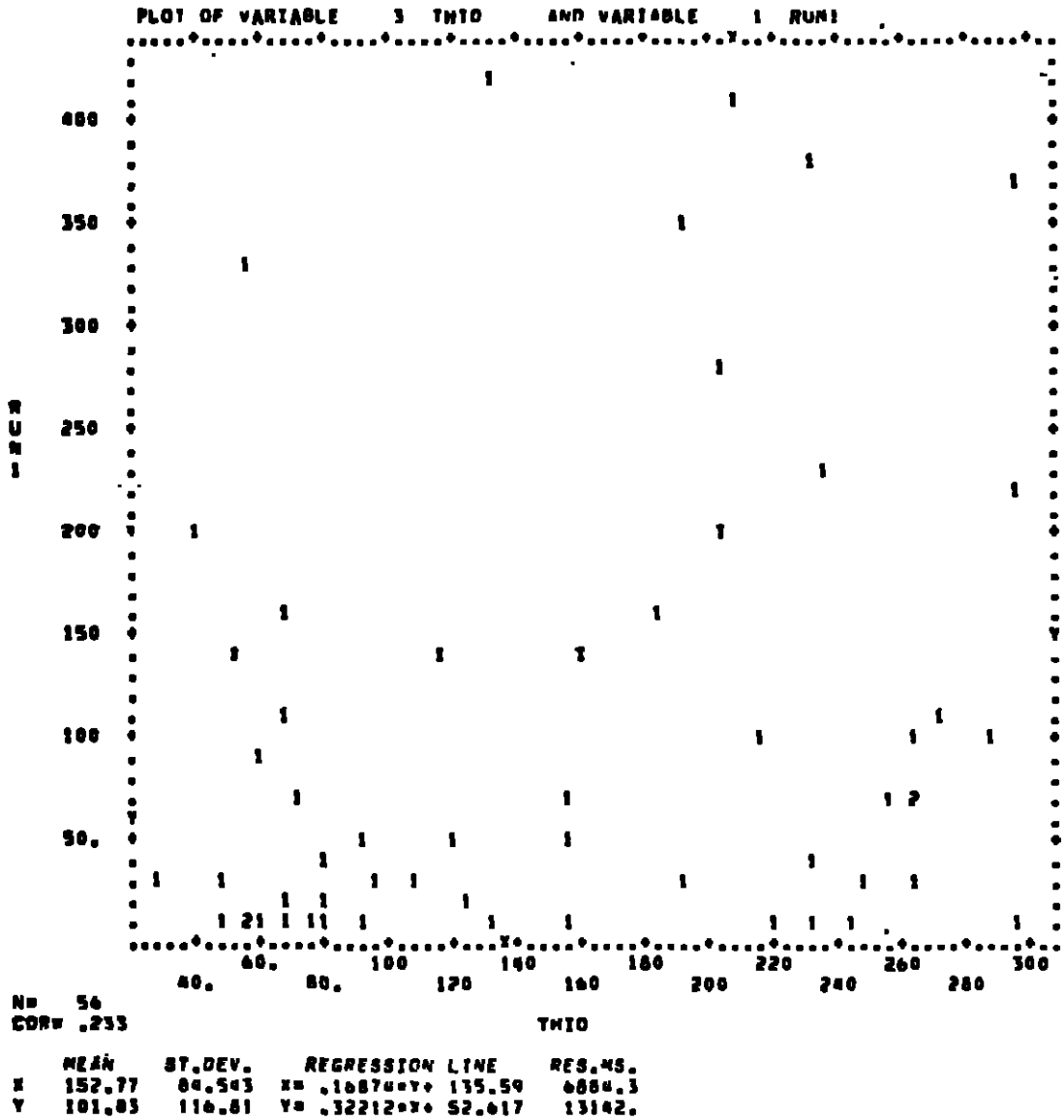


Figure 9. Scatterdiagram of Immunoglobulin A (trial 2) plotted against thiocyanate.

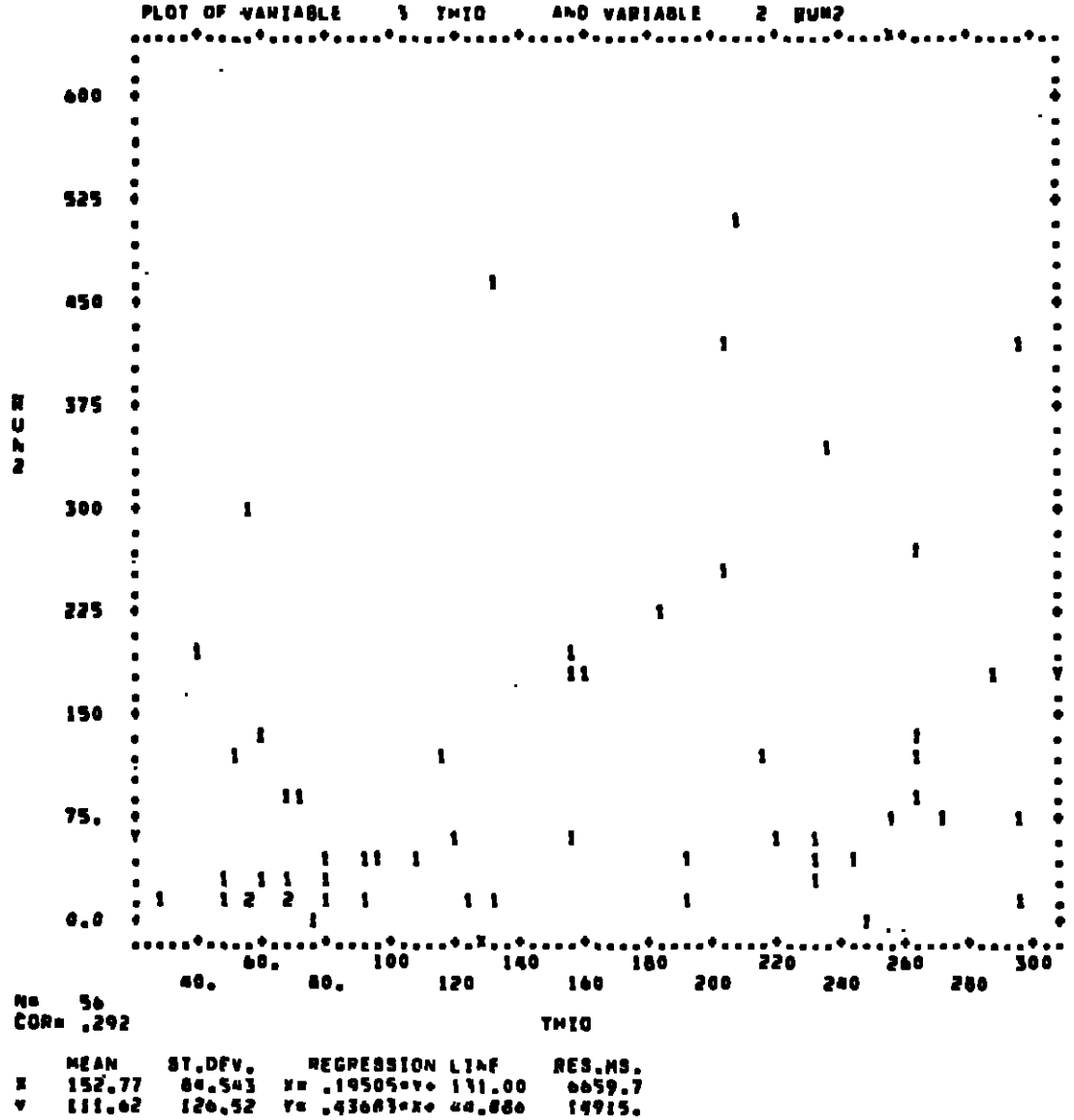
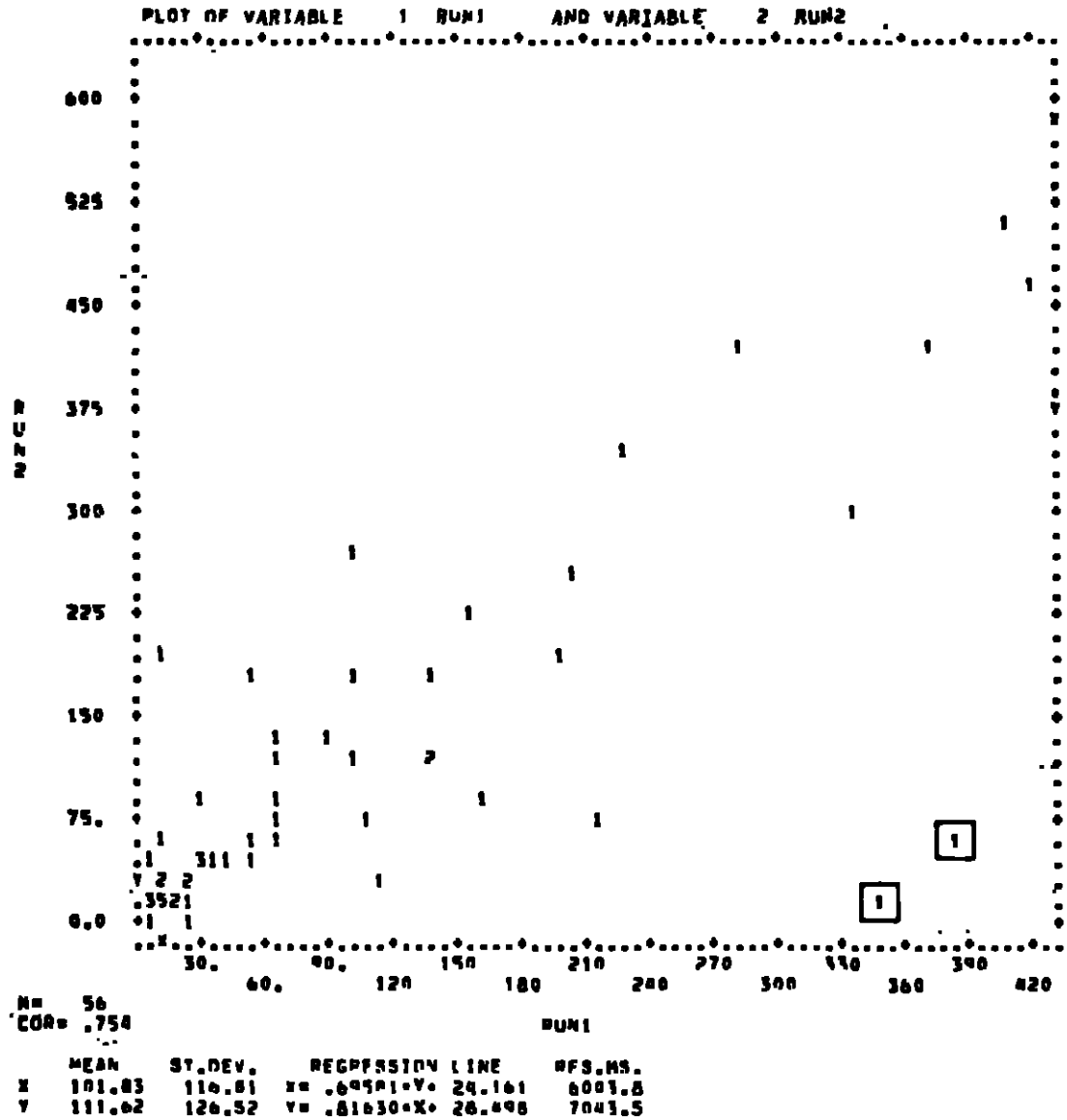


Figure 10. Plot of Immunoglobulin A (trial 1) vs. IgA (trial 2) to eliminate outliers.



were applied to the data. The natural log of IgA (trials 1,2) provided the most normal apportionment of the data. The descriptive statistics of the original experimental data such as analysis of central tendency, skewness, and kurtosis were applied to the transformed values and are presented in tables V and VI.

The normalization of the frequency distribution curve is presented in figures 11 and 12 for the natural log of IgA (trials 1,2). Scatter diagrams of this data plotted against thiocyanate can be observed in figures 13 and 14. Additionally, the correlated configuration between the two trials (without outliers) can be noted in figure 15.

Computations of Statistical Significance

The required normalization of the data and lack of significant linear correlation (Log Run 1) indicated that a more complex relationship (ex. curvilinear relationship) between IgA and thiocyanate may be present. Therefore, the independent variable, thiocyanate, was analyzed via a method of polynomial regression. Considering polynomial coefficients for thiocyanate of the fourth degree, the regression coefficient could be obtained and fitted to the log of IgA values. This data is presented in table VII. The multiple R-square term, .2276 or 22.76%, denoted the amount of variability of IgA accounted for by the fourth degree polynomial in thiocyanate. Considering the correlation of .900 between log IgA for each run as the

Table V. Statistical distribution analysis for the natural log of Immunoglobulin A (trial 1).

VARIABLE NUMBER	4	MAXIMUM	6.0426331
NUMBER OF DISTINCT VALUES .	41	MINIMUM	1.6094379
NUMBER OF VALUES COUNTED. .	56	RANGE	4.4331951
NUMBER OF VALUES NOT COUNTED	0	VARIANCE	1.6682552
		ST.DEV.	1.2916095
		(Q3-Q1)/2	1.0806948

LOCATION ESTIMATES		ST.ERROR
MEAN	3.9106615	0.1725986
MEDIAN	3.9796381	0.3171422
MODE	2.3025851	

```
            H       H          EACH 'H'
            H       HHH        REPRESENTS
            HH HH   HHH  H         1
          H HH HH HHHHH H      COUNT(S)
          H HHHHHHHHHHHHHH
          HHHHHHHHHHHHHHHH
         L----------------U

 EACH '-' ABOVE =     0.3000
             L=       1.2000
             U=       6.3000
```

	VALUE	VALUE/S.E.		
			Q1=	2.7658641
			Q3=	4.9272537
SKEWNESS	-0.04	-0.11	S-=	2.6190519
KURTOSIS	-1.19	-1.82	S+=	5.2022710

EACH '.' BELOW = 0.0500

```
                        S  Q                          Q   S
 M           M          -  1           M M            3   +             M
 I...........O.......................E.E..........................A
 N           D                       A D                                X
             E                       N I
```

VALUE	COUNT	PERCENTS CELL	PERCENTS CUM	VALUE	COUNT	PERCENTS CELL	PERCENTS CUM	VALUE	COUNT	PERCENTS CELL	PERCENTS CUM	VALUE	COUNT	PERCENTS CELL	PERCENTS CUM
1.60944	1	1.8	1.8	3.25810	3	5.4	35.7	4.61512	1	1.8	64.3	5.43808	1	1.8	87.5
1.70475	2	3.6	5.4	3.40120	3	5.4	41.1	4.62497	2	3.6	67.9	5.63479	1	1.8	89.3
2.05412	1	1.8	7.1	3.42100	1	1.8	42.9	4.69135	1	1.8	69.6	5.80814	1	1.8	91.1
2.14007	1	1.8	8.9	3.60021	1	1.8	44.6	4.72739	1	1.8	71.4	5.85793	1	1.8	92.9
2.30259	5	8.9	17.9	3.68888	1	1.8	46.4	4.90527	1	1.8	73.2	5.91350	1	1.8	94.6
2.42480	1	1.8	19.6	3.95124	1	1.8	48.2	4.92725	2	3.6	76.8	5.94280	1	1.8	96.4
2.59525	1	1.8	21.4	3.97029	1	1.8	50.0	5.05625	1	1.8	78.6	6.01616	1	1.8	98.2
2.68102	2	3.6	25.0	3.98898	1	1.8	51.8	5.06890	1	1.8	80.4	6.04263	1	1.8	100.0
2.85071	1	1.8	26.8	4.20469	4	7.1	58.9	5.29832	1	1.8	82.1				
2.86790	1	1.8	28.6	4.21951	1	1.8	60.7	5.30331	1	1.8	83.9				
3.19867	1	1.8	30.4	4.49981	1	1.8	62.5	5.37064	1	1.8	85.7				

Table VI. Statistical distribution analysis for natural log of Immunoglobulin A (trial 2).

VARIABLE NUMBER	5	MAXIMUM	6.2344108
NUMBER OF DISTINCT VALUES .	37	MINIMUM	1.5040774
NUMBER OF VALUES COUNTED. .	56	RANGE	4.7303333
NUMBER OF VALUES NOT COUNTED	0	VARIANCE	1.5299956
		ST.DEV.	1.2369299
		(Q3-Q1)/2	0.9599254

LOCATION ESTIMATES		ST.ERROR
MEAN	4.0617089	0.1652917
MEDIAN	4.0660596	0.2138905
MODE	NOT UNIQUE	

```
        M                   EACH 'M'
        MM                  REPRESENTS
    M   MM  M                   1
  M M MMMM M  M             COUNT(S)
 MMMM MMMM MM M
M MMMM MMMMMMMMM
MMMMMMMMMMMMMMMM
L---------------U
```

EACH '-' ABOVE = 0.3000
L= 1.5000
U= 6.6000

	VALUE	VALUE/S.E.		
			Q1=	3.1333632
			Q3=	5.0532141
SKEWNESS	-0.13	-0.40	S-=	2.8247790
KURTOSIS	-0.92	-1.41	S+=	5.2986388

EACH '.' BELOW = 0.0500

```
                     S   Q                        Q   S
M                    -   1          M             3   +            M
I...................................E...................................A
N                                   A                                  X
                                    N
```

VALUE	COUNT	PERCENTS CELL	CUM	VALUE	COUNT	PERCENTS CELL	CUM	VALUE	COUNT	PERCENTS CELL	CUM	VALUE	COUNT	PERCENTS CELL	CUM
1.50408	2	3.6	3.6	3.19867	1	1.8	26.8	4.40672	3	5.4	58.9	5.52146	1	1.8	87.5
2.05412	1	1.8	5.4	3.20275	1	1.8	28.6	4.49981	3	5.4	64.3	5.59099	1	1.8	89.3
2.30259	1	1.8	7.1	3.25810	2	3.6	32.1	4.72739	2	3.6	67.9	5.72359	1	1.8	91.1
2.39790	2	3.6	10.7	3.40120	1	1.8	33.9	4.74493	2	3.6	71.4	5.84354	1	1.8	92.9
2.42480	1	1.8	12.5	3.68888	2	3.6	37.5	4.92725	1	1.8	73.2	6.05678	2	3.6	96.4
2.56495	3	5.4	17.9	3.75887	1	1.8	39.3	4.98361	1	1.8	75.0	6.14204	1	1.8	98.2
2.77259	1	1.8	19.6	3.79549	1	1.8	41.1	5.16479	3	5.4	80.4	6.23441	1	1.8	100.0
2.86790	1	1.8	21.4	3.91202	3	5.4	46.4	5.28827	1	1.8	82.1				
2.93386	1	1.8	23.2	3.98898	2	3.6	50.0	5.29832	1	1.8	83.9				
3.06805	1	1.8	25.0	4.14313	2	3.6	53.6	5.40717	1	1.8	85.7				

HISTOGRAM OF VARIABLE 4 LRUN 1 Mean Std. Dev.

Symbol X Count 56 3.911 1.292

Interval Name	5 10 15 20 25 30 35 40	Frequency Int.	Frequency Cum.	Percentage Int.	Percentage Cum.
1.0000		0	0	0.0	0.0
2.0000	XXX	3	3	5.4	5.4
3.0000	XXXXXXXXXXXXXXXXXXXX	13	16	23.2	28.6
4.000	XXXXXXXXXXXXXXXXXXXX	13	29	23.2	51.8
5.000	XXXXXXXXXXXXXXXXXXXXX	14	43	25.0	76.8
6.000	XXXXXXXXXXXXXXXXXX	11	54	19.6	96.4
LAST	XX	2	56	3.6	100.0

5 10 15 20 25 30 35 40

Figure 11. Frequency Distribution of the natural log of Immunoglobulin A (trial 1).

HISTOGRAM OF VARIABLE 5 LRUN 2 Mean Std. Dev.

Symbol X Count 56 4.062 1.237

Interval Name	5 10 15 20 25 30 35 40	Frequency Int.	Frequency Cum.	Percentage Int.	Percentage Cum.
1.0000		0	0	0.0	0.0
2.0000	XX	2	2	3.6	3.6
3.0000	XXXXXXXXXXXXXXXXX	11	13	19.6	23.2
4.0000	XXXXXXXXXXXXXXXXXXXXX	15	28	26.8	50.0
5.0000	XXXXXXXXXXXXXXXXXXXX	14	42	25.0	75.0
6.0000	XXXXXXXXXXXXXXXXX	10	52	17.9	92.9
LAST	XXXXXX	4	56	7.1	100.0
	5 10 15 20 25 30 35 40				

Figure 12. Frequency Distribution of the natural log of Immunoglobulin A (trial 2).

Figure 13. Scatterdiagram of natural log of Immunoglobulin A (trial 1) plotted against thiocyanate.

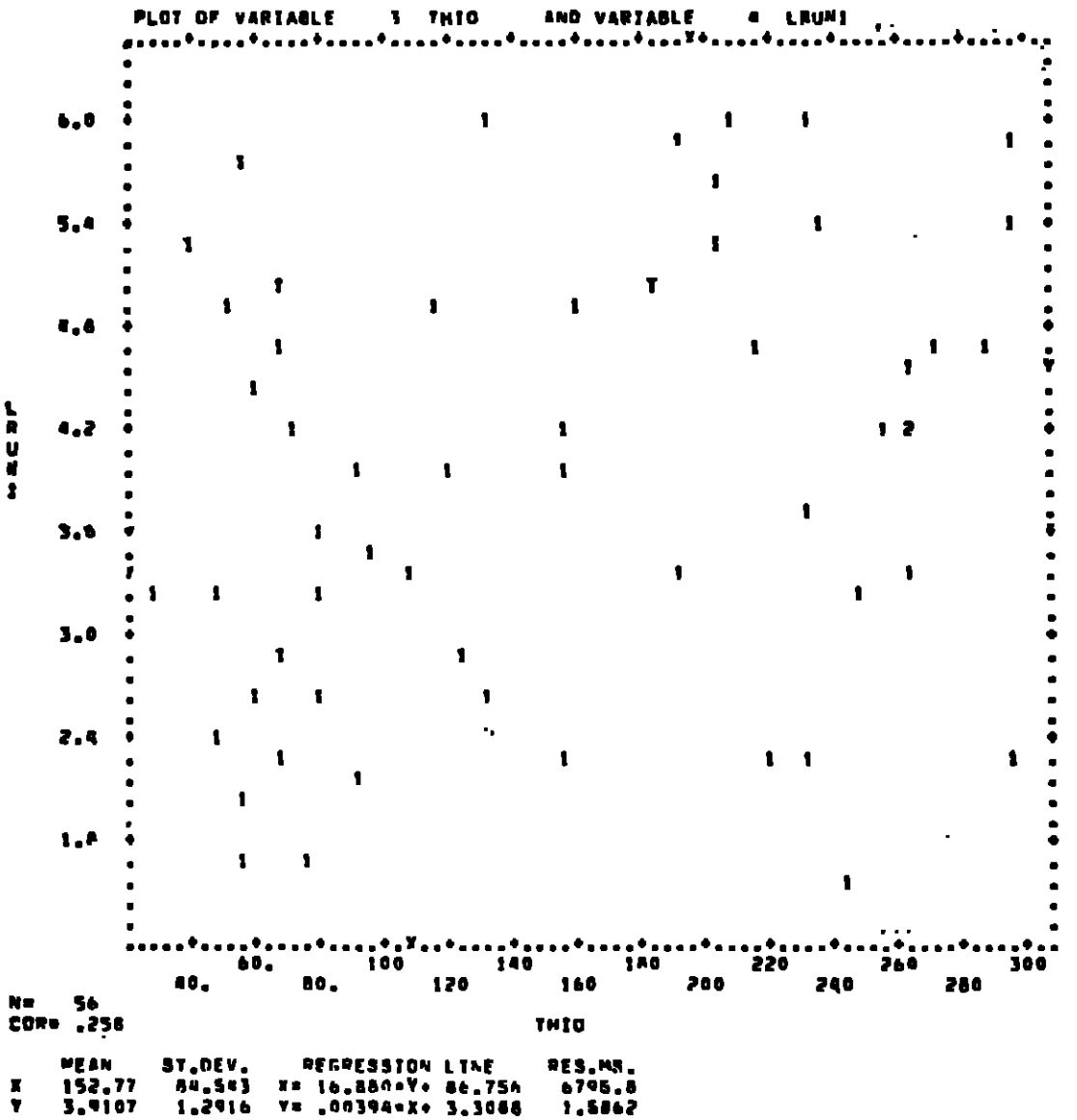


Figure 14. Scatterdiagram of natural log of Immunoglobulin A (trial 2) plotted against thiocyanate.

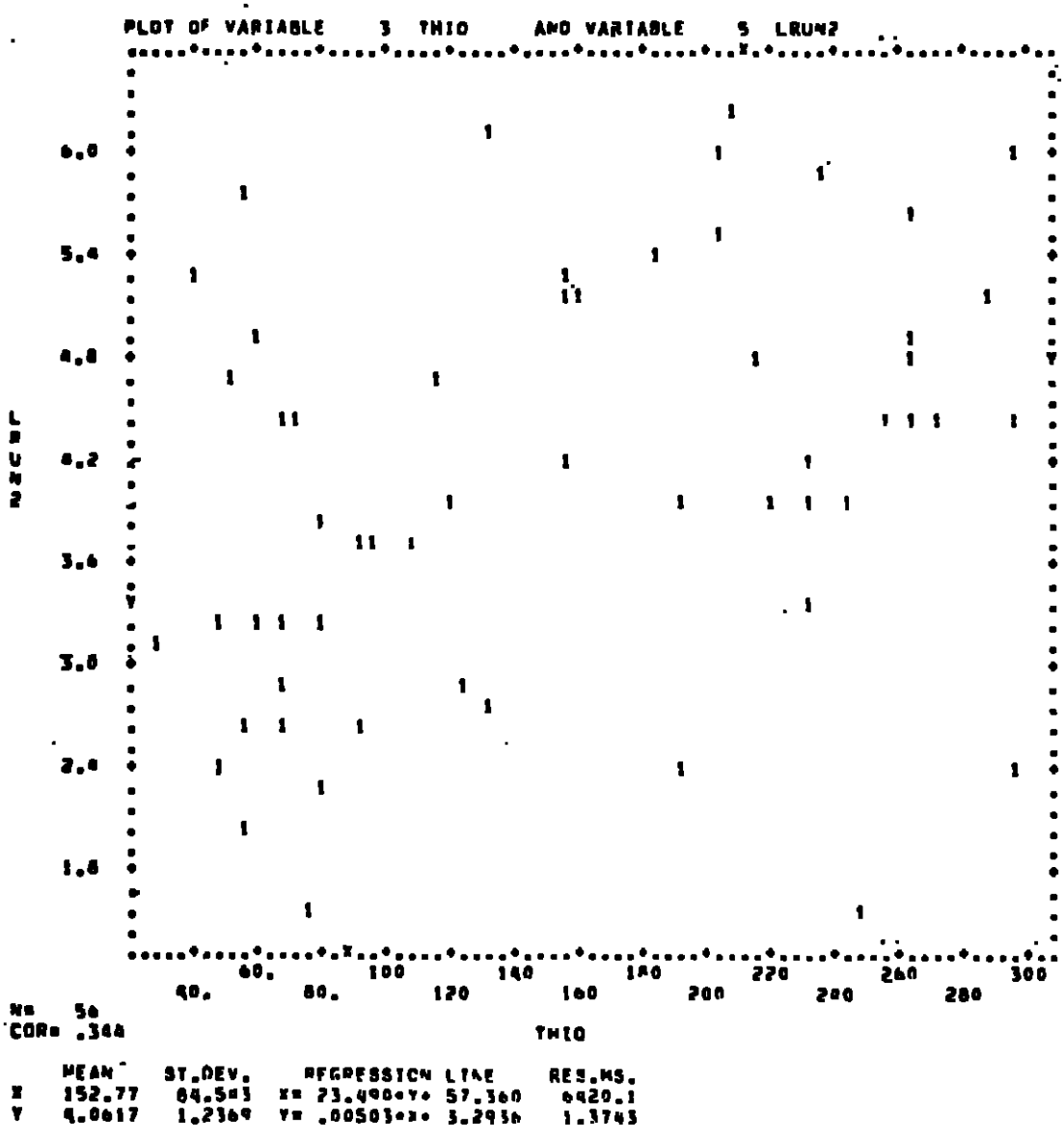


Figure 15. Natural log values of Immunoglobulin A (trial 1) plotted against the natural log of IgA (trial 2) without outliers.

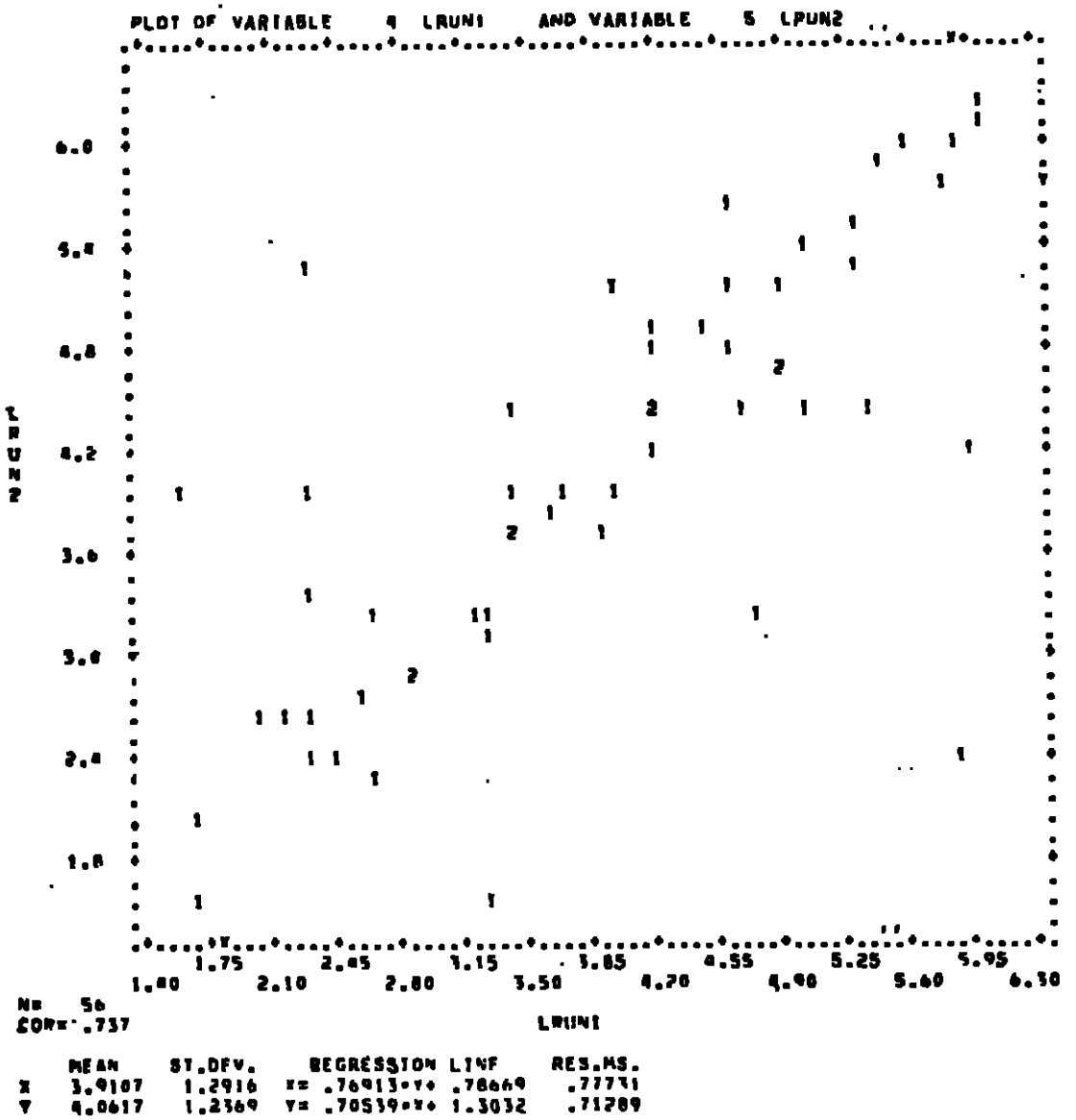


Table VII. Polynomial coefficients and significant regression data of the predicted curve.

POLYNOMIAL COEFFICIENTS — POLYNOMIAL IN X

DEGREE	REGRESSION COEFFICIENT	STANDARD ERROR	T VALUE
0	7.21658	2.36937	3.05
1	-14.28232	7.98560	-1.79
2	17.02891	8.85745	1.92
3	-7.32973	3.90267	-1.88
4	1.04942	0.59005	1.78

RESIDUAL MEAN SQUARE = 1.28196 (D.F. = 49)
MULTIPLE R-SQUARE = 0.22763

reliability of the measure of IgA (test-retest method) then the correlation for the unreliability in IgA (y) and thiocyanate (x) can be adjusted. The corrected correlation can be observed in equation 1,

$$r = \frac{\sqrt{r_{xy}}}{\sqrt{r_{yy}}} \qquad \text{Eq. 1}$$

where r_{xy} (the correlation between xy at .2276) and the correlation r_{yy} of reliability at .900 is equal to .503, which increased the multiple R-square to .253, or 25.30%. This represented the squared correlation of IgA with a fourth degree polynomial in thiocyanate if the error in IgA measure was eliminated.

The polynomial analysis of the log of IgA, thiocyanate, and the regression coefficients computed, were then incorporated into equation 2 to predict the curve.

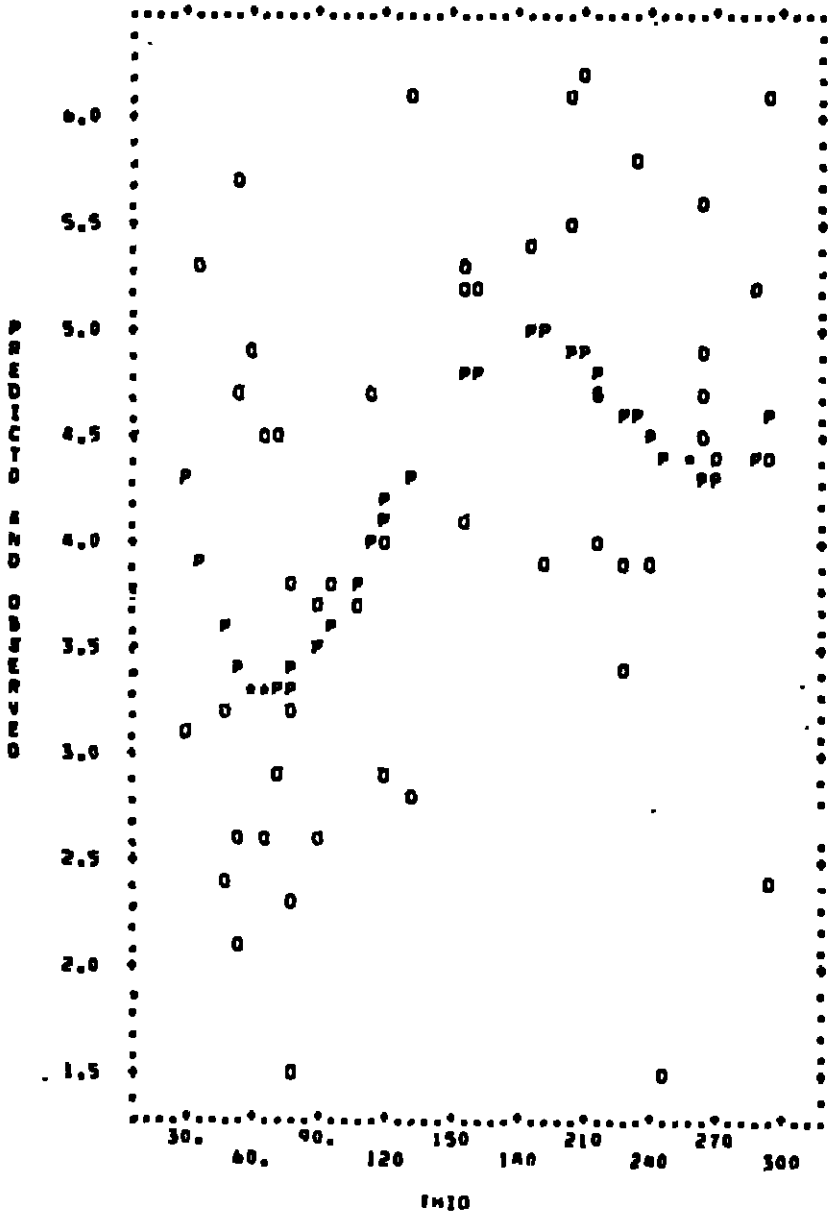
$$\log(IgA) = 7.22 - 14.28(T) + 17.03(T^2) - 7.33(T^3) + 1.05(T^4)$$

$$IgA = e^{\log(IgA)} \qquad \text{Eq. 2}$$

Substituting any value of T (thiocyanate) into the equation, the corresponding value of IgA could be obtained. Utilizing experimental thiocyanate values from 29 to 294 mg/ml, the predicted curve presented in figure 16 was achieved. This illustrated a cyclic relationship between the levels of IgA and thiocyanate ultimately related to increasing values of IgA assayed.

The frequency distribution of IgA for low and high

Figure 16. Observed values (O) of Immunoglobulin A plotted against thiocyanate with the predicted curve (P) indicated.



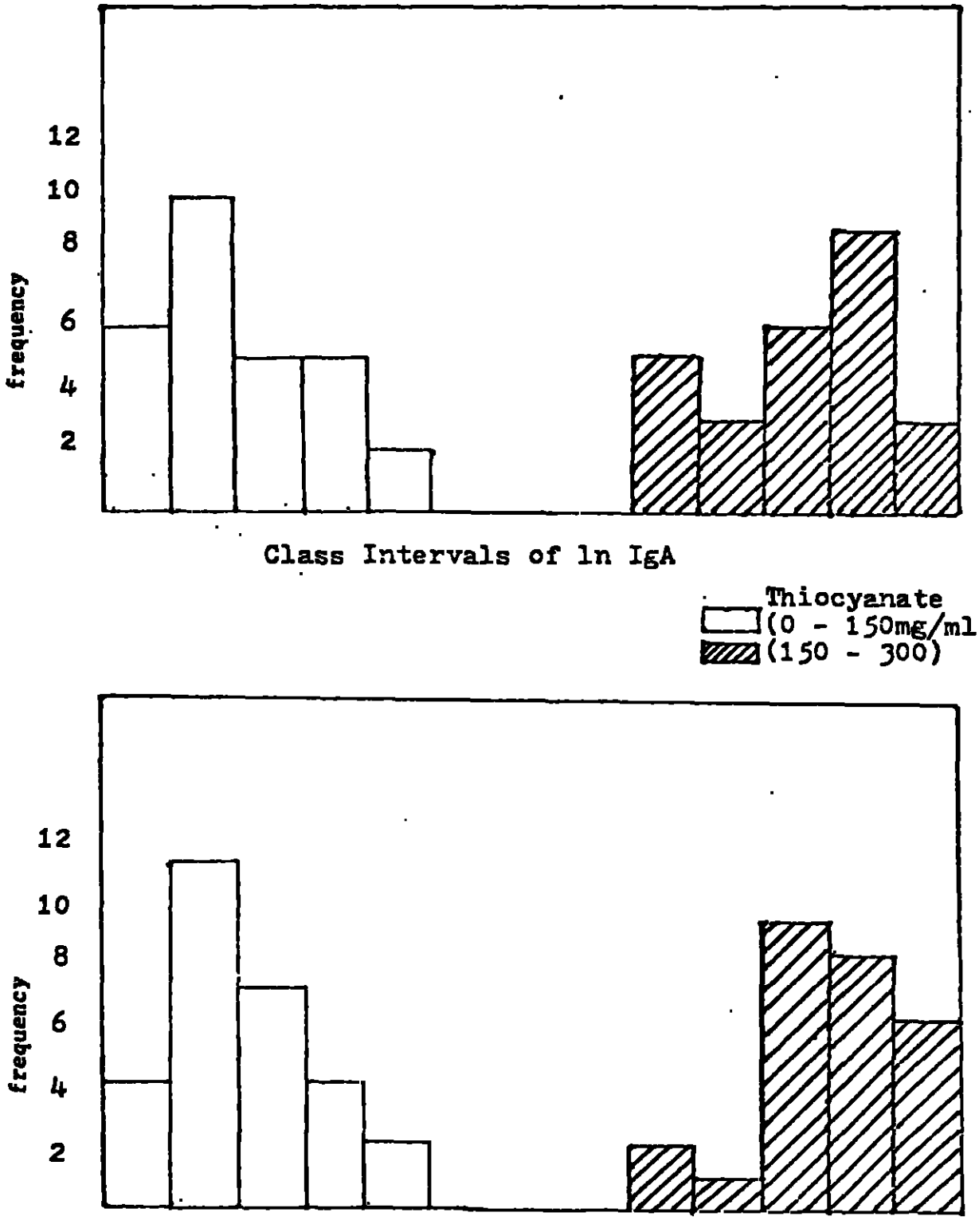
levels of thiocyanate were compared via a chi-square test. The histogram of IgA (trials 1,2 and 0-150 and 150-300mg/ml thiocyanate) appear in figure 17. The equation for chi-square calcuation is presented in equation 3.

$$x_4^2 = \frac{(y_o - y_e)^2}{y_e} \qquad \text{Eq. 3}$$

The observed (y_o) and expected (y_e) frequencies of IgA from the frequency distributions of figure 16 were substituted into the equation. The results indicate $x^2 = 5.29$, d.f. = 4 ($p > .05$) for trial 1 and $x^2 = 12.57$, d.f. = 4 ($p = .02$) for trial 2. Therefore, indicating that the frequency distribution between the IgA (0-150, 150-300mg/ml thiocyanate) were significantly different with less than 95% confidence for trial 1, but equal to greater than 98% confidence for trial 2. The distribution illustrated in figure 16 indicates this relationship.

The overall correlation of the experimentally obtained values of IgA (trials 1,2), thiocyanate, and the correlation displayed by the predicted model will be further discussed in the next section of this dissertation.

Figure 17. Comparision for frequency distributions of IgA with regard to thiocyanate levels.



CHAPTER IV

DISCUSSION

Statistical Analysis

Radial immunodiffusion techniques employed to quantitate the level of IgA in saliva produced concentrations within a range of 5-510mg/100ml. This is consistent with previous research (Brown et al, 1975; Aarli and Tonder, 1975). Additionally, protein levels determined by the Lowry (1951) method of protein estimation yielded a range of values from 5-102.5mg/ml. The mean and median values of total protein content showed marked decreases in samples containing increased IgA and thiocyanate levels. This information implies that IgA may be specifically increased for the increased thiocyanate containing samples.

Upon examination of IgA levels in relation to thiocyanate, linear regression proved inappropriate due to the lack of a normalized data distribution. Utilizing this method of analysis, the lack of significant correlation implied not only that no significant linear correlation existed, but also that a curvilinear or polynomial relationship may be present. This type of a relationship is less pronounced and generally portrayed by scattered

points such as those obtained in this analysis (Mendenhall and Reinmuth, 1978).

In order to pursue a higher level analysis, such as a multiple regression, the IgA data had to be normalized. This was accomplished via several reciprocal, square root and natural log transformations. The natural log of the IgA values achieved a close approximation to a normal distribution.

The relationship between the natural log of IgA (ln IgA) and thiocyanate was examined in second, third, and fourth order polynomial functions. Each of these generated a predicted "goodness of fit" to the data expressed as a correlation coefficient. However, it was not until the data was modeled using a fourth degree polynomial that the best fit was obtained. It represents a significant correlation of the complete quartic model rather than of the individual β parameters (polynomial coefficients examined singularly). The fourth degree polynomial gave a significantly better fit than the third degree. The individual regression coefficients just failed to reach significance, but this may be due to the multicolinearity inherent in a fourth degree polynomial.

This predicted model for the experimental data was further supported by equation 1 which expressed IgA concentrations as a function of observed thiocyanate concentrations via the coefficients for each polynomial, degree 0-4. Solving the equation for IgA with experimental

values of thiocyanate produced a quartic polynomial which closely approximated the predicted model.

Additionally, the chi-square analysis of the frequency distribution of IgA values (Eq. 2) produced $p > .05$ (trial 1) and $p < .02$ (trial 2). This predicted a less than 95% confidence (trial 1) and a greater than 98% confidence (trial 2) that the levels of IgA are dissimilar. When considering increased and decreased levels of thiocyanate with the correlation of the two trials at .900, the overall confidence is substantiated.

The variability of IgA described in the statistical analyses and illustrated in figure 17, demonstrated an increase in the level of IgA in response to increasing values of thiocyanate.

Significance of Analysis

The intent of this study was to test the effect, if any, of thiocyanate upon Immunoglobulin A in human saliva. Any variability in the levels of IgA may be postulated as a potential for variability in the acquired and innate protective immunity of secretions.

Experimentally, the concentration of the samples (via ultrafiltration and lyophilization), original sample volume, and degree of reaction in the RID plates, were all possible points of error. However, each area of experimentation received careful review to eliminate discrepancies in calculations and technique. Trials 1 and 2 demonstrated a

.900 correlation excluding outliers. This was the basis of all further statistical analysis.

The inherent areas of IgA variability such as genetic predispositon, infection, and allergic response, all contribute to altered levels of IgA in individuals and the general population. Additionally, the occurrence of human serum IgA, human secretory IgA, and associated proteins (secretory compoent and J-chain) further increased disparity of values between trials.

Personal habits may also have an effect on IgA levels in saliva. Food consumption, smoking, and drug intake may introduce unknown variability. Furthermore, IgA has been found to increase in the saliva of patients with oral cancer and also psoriasis. However, it appears to return to normal when the cancer is in remission (Brown et al., 1975; Oon et al., 1973).

More significantly, a study by Steigler and Citron (1974) demonstrated increased IgA concentrations in cases of mild or moderate bronchitis but decreased levels in patients with an advanced state of the disease. This may imply the existence of a cyclic IgA distribution such as that predicted by the current study. Moreover, the research of Siegler and Citron (1974), also reported the lack of secretory component in the advanced cases. This information, when combined with studies described by Plebani et al. (1979) of the effect of reducing drugs (ex. dithiothreitol) may be related to the results of the

current investigation.

Plebani et al. (1979) reported that dithiothreitol split sIgA from mucin, reduced polymeric sIgA to monomers and split the secretory component from sIgA. They further postulated that these physiochemical changes were likely to interfere with the normal protective function of IgA which acts as the first line of defense against foreign antigenic material and infectious agents.

Prior research by Aarli and Tonder (1975) demonstrated the decreasing effect upon IgA levels by anti-eptileptic drugs (ex. phenotoin) accompanied by increased levels of secretory component in the free unattached form.

The present study quantifies the levels of IgA when compared to thiocyanate. As indicated by the predicted model, IgA has a cyclic relationship with thiocyanate. Overall, 28.75% of its variability is accounted for exclusively by thiocyanate. Moreover, the increased values of thiocyanate indicate an increase in IgA values when examined by 0-4th degree polynomial equation.

Further examination of the fluctuating concentrations of IgA found in previous studies may indicate that the variability of IgA due to chemical factors may be cyclic in nature as demonstrated by this analysis.

Fluctuations in levels of thiocyanate as a result of various foodstuff ingestion or tobacco use as previously cited, has been shown to be associated with an appreciable percentage of the variability of saliva IgA levels. The

analysis of alterations in IgA levels, with regard to any deleterious effect to the individual, is not within the scope of this study. However, any change of secretory IgA could potentially alter the individual's capacity to resist encounters with certain disease producing entities.

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