pretreatment caused an increase in cardiac output subsequent to angiotensin, whereas nethalide caused a further reduction in this parameter.

From these results, it seemed apparent that, in addition to direct musculotropic effects, the neurogenic component of the angiotensin-induced pressor response may be mediated in part *via* the alpha adrenergic receptor.

SUMMARY

1. The injection of angiotensin into pentobarbital anesthetized animals resulted in an increase in systolic and diastolic pressures (with the latter of greater magnitude), right ventricular force, and coronary perfusion pressure. The increase in myocardial blood flow was of approximately the same magnitude as that of the diastolic pressure; however, angiotensin had no effect on coronary vascular resistance or heart rate.

2. Angiotensin elicited a biphasic response on the cardiac output of the animals. There was an initial decrease, followed by an apparent compensatory increase.

3. The above effects were mediated *via* both direct musculotropic actions and the sympathetic nervous system since acute surgical sympathectomy reduced, but did not abolish, the effects of angiotensin.

4. The sympathetic component of the cardiovascular effects of angiotensin appears to be *via* the alpha receptors since nethalide greatly potentiated the activity of angiotensin.

5. The effect of pretreatment with phenoxybenzamine, atropine, DCI, or nethalide on the cardiovascular responses to angiotensin are discussed in an effort to clarify its mechanism of action.

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Aspirin Formulation and Absorption Rate I

Criteria for Serum Measurements with Human Panels

By S. V. LIEBERMAN, S. R. KRAUS, J. MURRAY, and JOHN H. WOOD

Replication of serum salicylate level distribution in a human panel is obtained by rigorous adherence to properly selected protocols. The panel must be sufficiently large to represent all types of absorption variability after aspirin ingestion. The inherent variability of the human group can be measured. Thus, it is possible to calculate panel sizes required to provide the sensitivity, in terms of least significant differences, needed to evaluate factors of aspirin absorption. With the panels studied, random crossover testing offers no advantage over sequential testing. During the initial absorption period, serum salicylate levels existing at the time of ingestion, whether small artifacts or real and appreciable levels, may be treated as deductible blanks for the study of aspirin absorption.

IN RECENT medical and pharmaceutical literature there have been several papers related to some aspect of blood salicylate levels (1–10) or urinary recovery levels (11, 12) resulting from aspirin ingestion. There has been general agreement that the rate of absorption of drugs is reflected in the rate of increase of blood level and that in turn this level is reflected in the rate of urinary recovery. The results have not been in complete agreement, particularly on the blood salicylate levels resulting from different aspirin preparations; but not all the reasons for different levels have been established. The large varia-

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bility in early serum levels among individuals requires fuller consideration and a study of the factors which contribute to variability.

Some of the factors can be defined and standardized arbitrarily without experimental studies of alternative standardizations. These include a number of protocols for dealing with a panel of humans. Others can be standardized but require experimental justification. These include analytical procedures, treatment of salicylate levels encountered in serum blanks, and the selection from alternatives of two panel protocols, either random coadministration or sequential administration of test materials and either fasted or nonfasted subjects. A variability associated with the process of measuring aspirin absorption still remains, due to factors beyond simple definition or control by standardized procedures. This variability is amenable to statistical procedures, so that its magnitude and effect can be measured and considered.

This investigation has dealt with these factors; this and succeeding papers will establish the necessary protocols and criteria, study the *in vivo* absorption process as reflected by serum levels, develop suitable *in vitro* test procedures for various formulative changes, and provide suitable *in vivo–in vitro* correlations. With this basis, the role of additives in accelerating absorption will be examined for the mechanisms involved. In addition, the granulating and tableting procedures, press operation, role of excipients, disintegrating and wetting agents, humidity, and crystal habit of the aspirin will be examined for their relative contributions to better absorption. As a consequence of the correlations obtained, a general review of the kinetics of over-all drug absorption will be developed, including the relative contributions of *in vivo* dissolution, inherent absorptive process, hydrolysis kinetics, and clearance.

Relative to potent prescription drugs, aspirin is very innocuous. Therefore, it provides an excellent vehicle for an *in vivo* study of those factors which can affect the rate of drug uptake. Accordingly, much of the ensuing report should be considered as a general prototype of what may be done to study the absorptive capabilities in humans of any other drug with more restrictions.

It became apparent early in this study that the variability of *in vivo* measurements was greater than the variability of the *in vitro* measurements. Therefore, it was necessary to establish first a panel protocol to minimize variability and to determine a minimum panel size, so that the differences observed between two tests would be of high significance statistically. This paper reviews the problems of using the human panel, the precision of the analytical data, and the statistical treatment to delineate panel performance, so that the least significant difference for various tests with panels of different sizes could be determined.

TABLE I.—ANALYSIS OF SALICYLATE STANDARDS BY THE BRODIE PROCEDURE

Present, mcg.	Found, mcg.	Difference, mcg.
0.5	1.0	+0.5
1.4	1.5	+0.1
1.5	1.8	+0.3
2.6	2.0	−0.6
3.6	3.3	−0.3
3.9	4.0	+0.1
4.7	4.3	−0.4
8.7	7.5	−0.8
9.5	9.0	−0.5
19.5	17.3	−2.2
27.7	26.0	−1.3
48.7	51.0	+2.3

EXPERIMENTAL AND RESULTS

The blood samples were held at room temperature for a minimum of 2 hours to ensure complete hydrolysis of aspirin to salicylic acid. The analysis was by the Brodie procedure for serum levels (13, 14), modified slightly in transfer volumes to increase sensitivity. The volumes used were 2 ml. of serum mixed with 0.5 ml. of 6 *N* HCl and 30 ml. of specially purified ethylene dichloride for the first extraction. From this, 25 ml. of ethylene dichloride was taken for mixing with 5 ml. of ferric reagent. The color of the aqueous layer was then read on a Beckman DU

TABLE II.—INTERLABORATORY COMPARISONS OF SERUM SALICYLATE ANALYSES

Lab. *A* mcg./ml.	Lab. *B* mcg./ml.	Difference, *A*–*B* mcg./ml.
7.5	8.3	−0.8
7.5	8.0	−0.5
5.0	5.5	−0.5
4.0	4.5	−0.5
45.0	47.0	−2.0
1.5	3.5	−2.0
23.0	25.0	−2.0
4.0	5.3	−1.3
55.0	54.5	+0.5
7.5	7.5	0.0
62.5	68.0	−4.5
15.2	15.0	+0.2
36.0	32.0	+4.0
51.0	48.6	+2.4
14.0	11.8	+2.2
5.5	3.0	+2.5
27.5	28.0	−0.5
27.0	26.8	+0.2
7.5	8.0	−0.5
7.0	7.5	−0.5
27.0	28.5	−1.5
53.5	56.0	−2.5
28.5	30.0	−1.5
18.0	18.0	0.0
45.0	44.8	+0.2
18.0	21.0	−3.0
23.0	22.0	+1.0
55.5	53.0	+2.5

TABLE III.—SALICYLATE SERUM LEVELS (mcg./ml.) FOR SIX SAMPLES ADMINISTERED REPETITIOUSLY TO THE PANEL

Aspirin Prepn.	Test Code	Mo./Yr.	No.[a]	10-Min. Levels $\bar{x}$[b]	10-Min. Levels S.D.[c]	20-Min. Levels $\bar{x}$	20-Min. Levels S.D.
1	L	3/58	40	6.92	5.99	18.14	11.34
1	SC	3/59	39	7.69	6.16	18.71	9.95
2	EA	3/58	40	4.89	6.19	15.67	14.25
2	ER	3/58	39	5.39	7.30	14.78	14.52
3	AA	3/58	40	9.25	8.70	30.33	17.09
3	AR	4/58	39	10.47	12.17	27.87	17.89
4	BD	10/58	35	10.21	9.26	32.64	16.81
4	BF	11/58	39	14.37	13.33	32.17	19.29
5	BE	10/58	37	14.24	12.19	34.59	16.54
5	BG	11/58	38	14.67	13.55	35.53	16.51
6	SA	10/58	42	4.63	5.29	16.66	12.57
6	SB	2/59	39	8.10	9.23	20.15	15.58
6	SX	3/59	38	7.21	9.55	17.01	15.14
6	SY	3/59	39	7.43	6.84	21.55	12.44
6	SZ	1/60	39	9.19	7.67	21.10	11.36

[a] Number of subjects. [b] $\bar{x}$, Average level. [c] S.D., Standard deviation of individual observation.

spectrophotometer against a reagent blank. Analysis results for a series of standards in serum carried through the procedure are given in Table I. Analyses were performed in two different laboratories. The data for interlaboratory comparisons on random samples run during normal routine in both laboratories are given in Table II.

The serum blank usually ranged from zero to a small finite value. In all work reported the serum blank was subtracted from the observed readings of that individual. In one test, the panel was predosed with 10 gr. of aspirin 10–12 hours prior to the test to provide large finite zero time levels for all subjects. These large serum blanks were subtracted. The results are given in Table III, and the analyses for individual paired differences with (test SX) and without (test SB) predosing are given in Table IV.

The subjects ate normal light breakfasts at home about 2 to 3 hours before the test and took nothing in the interim. For one test (SY) of a reference lot of tablets, they were strictly fasted after the previous evening meal. This result (Table III) was compared (Table IV) to test SB of the same reference lot and employed normal panel conditions.

The panel was used over a long term, with infrequent changes in personnel, for sequential testing. Tests of reference and some other tablets were repeated at intervals as checks during the course of the investigation. The results and comparisons are listed in Tables III and IV. Tests SA, SB, SX, SY, and SZ are repetitions of one reference lot of tablets, although there were special conditions with test SX (predosed subjects for high initial level) and test SY (fasted subjects). There were a number of duplicate tests: EA and ER, AA and AR, BD and BF, BE and BG, and L and SC. Some were preparations of aspirin alone, and some had an antacid in the tablet. Six formulations are involved; these are identified by number to show replications in Table III. All tests have involved ingestion of two tablets, each containing 5 gr. of aspirin.

Because of economy of time and effort, sequential testing was the procedure chosen; a comparison of this procedure to random coadministration procedure was obtained by scheduling four of the desired repetitions as two pairs of random coadministration tests. Tests AR and ER were paired random coadministration repetitions of sequential test AA and EA, respectively. Tests BF and BG were sequential repetitions of BD and BE, respectively, done earlier as a random coadministration pair.

To obtain a least significant difference smaller than that observed with other panels, our initial panels were 30 subjects per test, increased to 40 later. The data required for computing statistical values are included in Table III.

A number of protocols were fixed at the outset and have been continued with no further consideration. Subjects were paid volunteers selected only for their availability; participation by subjects was weekly, using a fixed day and hour for each subject. There was infrequent rescheduling; if a subject had to be scheduled for a second participation within 1 week,

TABLE IV.—STATISTICAL DATA FOR COMPARISONS OF TESTS FOR TABLE III BY PAIR DIFFERENCES

Test Codes	No.[a]	10-Min. Levels $\bar{d}$	10-Min. Levels S.D.[b]	10-Min. Levels t[c]	20-Min. Levels $\bar{d}$	20-Min. Levels S.D.	20-Min. Levels t
SB–SX	37	0.62	10.56	0.36	3.20	17.08	1.14
SB–SY	38	0.05	8.66	0.035	−2.06	16.26	0.78
EA–ER	39	−0.39	7.81	0.31	1.18	13.92	0.53
SA–SB	37	−3.44	9.80	2.13	−3.55	15.31	1.41
SB–SZ	31	−2.22	8.50	1.46	−3.37	13.96	1.35
AA–AR	39	−1.17	13.29	0.55	2.56	15.77	1.01
BD–BF	30	−3.45	17.53	1.09	2.72	25.52	0.59
BE–BG	29	1.15	15.00	0.40	1.78	21.38	0.45
(BD–BE)-(BF–BG)	28	−4.23	23.1	0.971	2.95	30.0	0.521
(ER–AR)-(EA–AA)	39	−1.38	16.5	0.523	1.40	21.4	0.407

[a] Number of individuals participating in *both* tests for whom data were paired. [b] S.D., Standard deviation of individual pair differences. [c] t, Student t value for the null hypothesis.

the minimum interval was 3 days. All were instructed to avoid medications for 3 days prior and fatty foods for 1 day prior to preclude analytical difficulties caused by interference or by cloudy serum. They were questioned routinely on these points. Rescheduling for any reason entailed no financial penalties, and cooperation was excellent. The sampling time was the time of stopping blood circulation in the arm with a cuff, with 10–12 ml. of blood withdrawn promptly (usually within 30 seconds) by venipuncture in the cubital area. Prior to test ingestions, a blood sample was drawn to provide a (zero time) serum blank. The test tablets were taken with 100 ml. of room temperature water.

DISCUSSION

The precision and accuracy of the slightly modified analytical procedure were considered adequate for the measurements to be made. The initial trial of recovery of known amounts added to serum, shown in Table I, was evaluated by regression analysis. For these 12 pairs, the slope of the line was 1.02, not significantly different from 1; its intercept was −0.52, not significantly different from zero. Two laboratories were involved in analyzing serum samples—simultaneously at one time and singularly at other times. Some blood samples, randomly designated, were drawn to provide sufficient serum for both laboratories at the rate of four per week for 7 weeks, with one analyst unaware of the checking, and the second analyst aware of his function. The 28 results are shown in chronological sequence in Table II. The correlation coefficient was 0.995, the slope of the line 1.01, and its intercept 0.09. These do not differ significantly from unity and zero, respectively. For a group of such data containing a number of items, each with its slight error, the statistical treatment shows that the standard error of the average will be small.

The human variability makes the major contribution to total variability. This is so large that only statistical parameters can represent the data adequately. Examination of serum salicylate levels for individuals in repetitive tests shows ranges varying from small for some subjects to large for others. There are some people who show small variabilities between tests at the low, intermediate, and high levels. There are others whose variability is so great that it is impossible to assign them to a level category. The variability within a group is great. The distribution of the levels with a panel for any one test substance is somewhat skewed from the normal. The skewness is less apparent with higher average levels, but is remarkably reproducible in panels of sufficient size, even though individual variability is high. Figure 1 shows the shape and reproducibility of distributions of levels for repetitions of the same preparation under codes SA, SB, and SZ. The reproducibility of the distributions is also evident in the reproducibility of the averages and medians (Table III). Since the population of levels is a skewed distribution, there is the choice of a suitable measure of the panel. The average is, of course, the most readily determined parameter, but it is the most sensitive to a few outlying values in the distribution. The median is another useful parameter for comparisons. Approximately two-thirds of the population have the average level or lower in

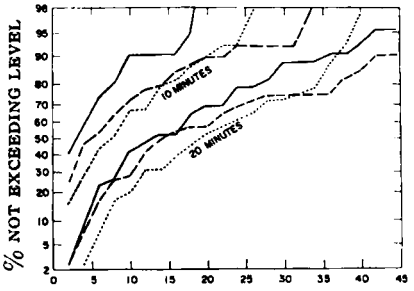

Fig. 1.—Cumulative per cent plots on probability scale for aspirin preparation 6 studied three times (panel essentially invariant). Key: ——, SA; - - -, SB; · · ·, SZ.

time periods reflecting the early absorption process. The use of distributions and medians in assessing test results can render an insight into affects of formulation and processing changes but lacks the utility of averages and standard deviations in statistical comparisons.

In comparing two tests, the Student t test can be applied to the difference between the two averages, using a standard deviation resulting from pooling the two variances. Another procedure is available if the same panel subjects were used in both tests. The average and its standard deviation of the individual differences between the two tests (pair differences) can be used in the t test to measure the significance of difference from zero of the average pair difference. Depending on the variances and correlation coefficient encountered, either procedure may be favored in various circumstances. In this paper, the method of pair differences has been chosen arbitrarily for all comparisons; these appear in Table IV.

The magnitudes of the variations within and between individuals for a useful differentiation during early absorption require a panel larger than has been used in previous studies. The smaller panels suffered from the disadvantage of not representing the categories now observed in the population. The ability to differentiate two test products is related inversely to the standard deviation of average paired differences and directly with the square root of the size of the panel. Finer differentiations require either reduced standard deviation or more subjects.

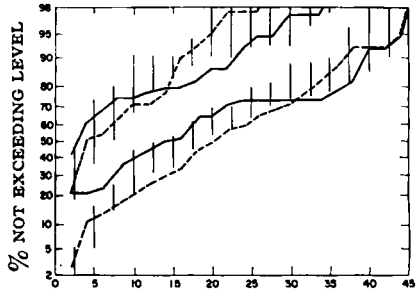

Fig. 2.—Cumulative per cent plots on probability scale resulting from subtraction of large zero levels (SX, ——) and for complete fasting (SY, - - -). Vertical lines indicate range of normal repeats from Fig. 1.

Only the latter can be manipulated, and the number selected controls the least significant difference (l.s.d.) attaining statistical significance. After the first panels of 30, a panel of 40 was considered optimum for balancing the volume of work with the ability to differentiate. This size, with individuals occasionally unavailable plus panel membership attrition, permitted a sufficient number to be available for the method of paired differences to give the desired l.s.d. Thus, in Table V are tabulated average l.s.d. for a 95% confidence for typical 10- and 20-minute levels for slowly and rapidly absorbed products. These are calculated for panels of various sizes using representative standard deviations observed for the panels and the appropriate Student t value (15). The l.s.d. for other confidence levels may be calculated similarly. Note that Table V refers to pair differences. For comparison of data from two different panels, a pooled standard deviation is required, and the l.s.d. is increased further by the square root of 2.

During the early absorption from tablets, the standard deviations for the individual runs or for the paired results for the several duplicates show similar magnitudes for corresponding averages. This similarity in magnitude is purely coincidental, and no

Table V.—Least Significant Difference for Pair Data (95% Confidence) Resulting from Panel Size at Three Typical Average Salicylate Levels

Panel, No.	l.s.d. $\bar{x}$[a] = 7 mcg./ml. (S.D.[b] = 8)	l.s.d. $\bar{x}$ = 17 mcg./ml. (S.D. = 13)	l.s.d. $\bar{x}$ = 32 mcg./ml. (S.D. = 18)
3	19.9	32.3	44.7
5	9.1	16.1	22.3
10	5.7	9.3	12.9
20	3.7	6.1	8.4
30	3.0	4.8	6.7
40	2.6	4.2	5.9
50	2.3	3.7	5.1
100	1.6	2.6	3.6

[a] $\bar{x}$, Average serum salicylate level at 10 or 20 minutes. [b] S.D., Typical standard deviation of average level (taken from Fig. 3).

significance should be drawn. It has been noticed that the standard deviation appears to increase with an increase in average blood levels during the absorption period. Any test shows both fast and slow absorbers giving high and low levels; but as the average level increases, the proportion of high levels increases at the expense of the number of slow absorbers. The result is an increase in variance as average increases (Fig. 3) during early absorption. The time and serum level dependency of variance will be treated in the next paper.

An examination of the data for tests L and SC in Table III shows the closeness of agreement in averages. This is especially evident in Fig. 4 which gives the two distributions involved, an indication that if the group is sufficiently large, data replication dose not require all individuals common to both tests. There were only 25 subjects common to test L and SC. The pair comparison is omitted from Table IV.

Reproducibility of results with the panel of subjects was achieved over a long period. Table IV shows repeat runs made 1 week and 1 year apart and

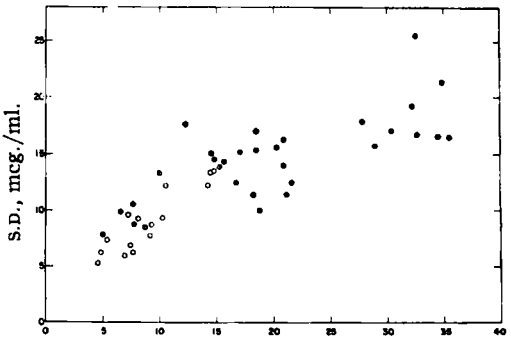

Fig. 3.—Variation of standard deviations with salicylate serum levels after absorption. Key: O, 10-minute average and ●, 20-minute average level; ◐, 10-minute pair difference and ◑, 20-minute pair difference plotted at the average of the pair.

some intermediate intervals. Credit for this achievement must go to rigid adherence to the fixed protocols to limit the experimental variability and the use of sufficient numbers of subjects to cope with human variability. There are eight pair difference comparisons in Table IV of both 10- and 20-minute serum levels. The pairs are each duplicate tests, and only one of the 16 comparisons has a significant t value. With 16 comparisons expected to be nonsignificant differences at 95% confidence, the discovery of one contrary to expectations is not surprising.

Early consideration for conducting the program as either a series involving coadministration of multiple test materials with random sequence assignments, or a series of sequential single tests, favored the latter. This offers economy of time and effort with less repetitions of standards and, with *rigid adherence* to protocols combined with only slight changes in panel personnel, no loss in sensitivity of measurements. The former procedure (random crossover) has a wide vogue in biological testing. Rigid adherence to protocols is the only protection against the occasional unrecognized error by either mode. It was possible to schedule a number of the wanted repetitions, so that both procedures were utilized and a comparison became available. Four of the comparisons given in Table IV (EA–ER, AA–AR, BD–BF, and BE–BG) had one test of each pair performed as a single test in the long sequential series.

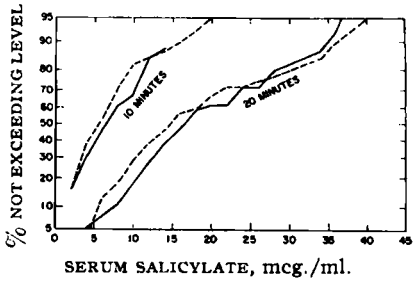

Fig. 4.—Cumulative per cent plots on probability scale for aspirin preparation 1 administered as L (- - -) and SC (——). (Only two-thirds of panel common to both tests.)

The four other tests of these four pairs were performed as two pairs of random crossover tests. None of the eight comparisons (four with 10-minute data and four with 20 minute data) performed as paired differences for individual subjects showed a significant difference from zero for the average pair difference. Another comparison is possible: that of the average individual difference between the two tests of the crossover period and the corresponding two tests of the sequential series—BD–BE of the crossover with BF–BG of the sequential and EA–AR of the crossover with EA and AA of the sequential. Table IV shows that none of the average differences were significantly different from zero. With both kinds of comparisons, the results of the carefully controlled sequential procedure were not different from the random crossover procedure.

Serum blanks (zero time samples) often exhibit low salicylate values which may be *bona fide* or artifacts. Occasionally, a subject showed a high zero time level which could be traced to recent ingestion of medications containing salicylates and, in one case of serendipitous sleuthing, wintergreen (methyl salicylate)-flavored candy lozenges. For some time, all data for subjects with a high zero time level were rejected, and the subject was rescheduled. The feasibility of subtracting serum blanks was demonstrated in one test (SX). All subjects were given two tablets (10 gr. of aspirin) to take the night before their scheduled test. As a result, everyone appeared with a high serum blank value which averaged 14.1 mcg./ml., with a range from 3.5 to 52.3. On correcting the data by subtracting the appropriate serum blanks, the 10- and 20-minute levels were like the results of test SB using the same tablets. For test SB, however, the serum blanks averaged 1.7 mcg./ml., with a range from 0.5 to 3.5. Subtraction of serum blanks of any level is now routine, although the injunction against medication during the 3 days prior is continued, and high serum blanks are rare. Where the duration of the test will result in any natural decay of the blank level, a correction for the clearance rate (16) is required.

Prior to initiation of this work in 1957, discussions with clinicians indicated a consensus that higher blood levels and more consistent results were expected for a fasting panel. Necessary to the reproducibility of the panel was the need for careful control and constancy of extraneous background. Figure 2 and Table IV, in which the panel distribution of a truly fasted panel (SY) is compared to the normal (SB), show that statistically no difference existed between the normal (semifasted) and fasted panel. A more positive assurance for this one test of a fasted panel is the observation that the distribution curves (Fig. 2) are obvious members of the family of curves from other tests of the same aspirin tablets. Thus, by waiting 2 to 3 hours after a light breakfast, it is possible to have the test advantage of the fasted panel without the personal disadvantages of periodic fasting over a long term.

SUMMARY

A modified Brodie serum salicylate analysis has been shown to have the necessary precision and accuracy for following serum salicylate levels in groups of 30 to 40 subjects.

Human variability is of such magnitude that the data are represented fairly by statistical parameters only.

Rigid compliance with proper protocols and large panels of subjects give statistically reproducible average blood levels.

The least significant difference, useful in differentiating test results, is controlled primarily by the number of subjects.

Carefully controlled sequential testing with adequate panels gives results indistinguishable from those obtained by random crossover testing. Panel replicability over prolonged periods is excellent and defined by statistical parameters.

Serum blanks, or zero time serum salicylate levels, can be subtracted from the serum levels of subsequent samples for 10- and 20-minute intervals.

A panel of semifasted subjects is as useful in results as a panel of fasted subjects and more amenable to a long-term testing program.

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