Blood pyruvate concentration measured by a specific method in control subjects

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SYNOPSIS   A specific method of estimating pyruvate, depending upon the oxidation of reduced nicotinamide-adenine dinucleotide in the presence of lactic dehydrogenase, has been modified to improve its accuracy. The precision of the modification, measured by duplicate analysis, was represented by a standard deviation of 0·03 mg./100 ml. The mean recovery of added pyruvate was 99%.
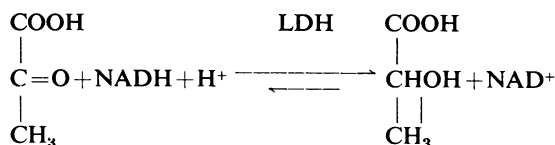
The mean blood pyruvate concentration in 30 fasted subjects, who had no illness known to affect pyruvate metabolism, was 0·44 mg./100 ml. (S.D. 0·05). Blood pyruvate levels were measured at 30-minute intervals for two hours after oral or intravenous administration of glucose. The highest levels obtained in each case were about 0·4 mg./100 ml. higher than the initial level, and were found 60 to 90 minutes after oral administration and 30 to 60 minutes after intravenous administration.

Pyruvic acid occupies a central position in intermediary metabolism, and its level in the blood is affected by many conditions. It is important that a specific and relatively rapid method for its measurement in biological solutions should be available.

The most commonly used methods of measuring pyruvate depend upon its condensation with 2,4-dinitrophenylhydrazine. Several modifications have been developed (Barrenscheen and Dreguss, 1931; Lu, 1939; Bueding and Wortis, 1940; Klein, 1941; Bonting, 1955), the most popular being that of Friedemann and Haugen (1943). These methods, however, are not specific because the results are variably affected by other α-keto acids.

Paper chromatography of the hydrazones of the α-keto acids was introduced by Cavallini, Frontali, and Toschi (1949) for the specific measurement of pyruvate and has been used by several other workers, including Seligson and Shapiro (1952), El Hawary and Thompson (1953), and McArdle (1957). This step, however, is time consuming and may result in loss of some of the pyruvic acid hydrazone.

Specific and more rapid methods for measuring pyruvate have been introduced. These depend upon the oxidation of reduced nicotinamide-adenine dinucleotide (NADH) in the presence of lactic dehydrogenase (LDH) according to the following equation:

$$\underset{\substack{|\\ CH_3}}{\overset{\substack{COOH\\ |}}{C=O}}+NADH+H^+ \xrightarrow[]{LDH} \underset{\substack{|\ |\\ CH_3}}{\overset{\substack{COOH\\ |}}{CHOH}}+NAD^+$$

The oxidation of nicotinamide-adenine dinucleotide is measured spectrophotometrically by the change in extinction at its peak absorption of 340 mμ. The reaction between pyruvic acid and reduced nicotinamide adenine dinucleotide is stoichiometric. Since the equilibrium of this reaction lies far to the right (Kubowitz and Ott, 1943), the fall in extinction is proportional to the pyruvate concentration. Hess (1955) added reduced nicotinamide-adenine dinucleotide to serum and made use of the serum lactic dehydrogenase present. Other workers (Bauer, 1956; Redetzki, Bloedorn, and Bansi, 1956; Segal, Blair, and Wyngaarden, 1956) deproteinized blood immediately to inactivate its lactic dehydrogenase, and later established a buffer system with a *p*H of about 6 and added reduced nicotinamide-adenine dinucleotide and then lactic dehydrogenase to promote the reaction.

The purpose of the present paper is to report some observations on the nicotinamide-adenine dinucleotide reaction for the measurement of blood pyruvic acid. The levels in control subjects while fasting and after oral or intravenous administration of glucose are also recorded.

SUBJECTS Thirty subjects, 14 men and 16 women, between 16 and 74 years of age, were studied. None had any illness known to affect pyruvate metabolism, and all were ambulant and received an adequate diet.

PROCEDURES The following procedures were carried out after a fast of from 14 to 17 hours:—

Estimation of fasting levels of blood pyruvate and investigation of effect of oral glucose on blood pyruvate concentration A 50% solution of glucose (1 g./kg. body weight) was given orally, and blood samples were taken at 30-minute intervals for two hours for the estimation of blood pyruvate and blood sugar levels.

Investigation of effect of intravenous glucose on blood pyruvate concentration A 50% solution of glucose (0·5 g./kg. body weight) was injected intravenously within four minutes. Blood samples were taken at 30-minute intervals for two hours for the estimation of blood pyruvate concentration and at 15-minute intervals for the estimation of blood sugar.

Blood was taken from a vein in the forearm or antecubital fossa, usually through an indwelling needle. When an indwelling needle was used, a solution of heparin and saline was introduced between sampling to prevent clotting. The heparinized saline solution was washed out of the needle with 1 ml. of blood immediately before taking each sample.

Blood samples were not taken until several minutes after inserting the needle. The effects of hand warming, hand clenching, and venous occlusion were studied. In investigations an adequate blood flow was encouraged by warming the hand with an electric pad, hand clenching and venous occlusion being avoided.

METHOD

About 4 ml. of blood was withdrawn rapidly and transferred to a tube containing heparin. Three ml. of blood was then pipetted slowly, continuously mixing with a small rod, into 6 ml. of 0·6 M perchloric acid in a centrifuge tube surrounded by ice and water. Manipulations were adjusted so that the interval between the midpoint of withdrawing the blood and the midpoint of adding it to the perchloric acid was as close as possible to one minute. The tube was then sealed with parafilm and the contents mixed carefully by inversion. The tube was kept at a temperature of 0 to 5°C until it could be centrifuged at about 3,000 r.p.m. for five to 10 minutes.

A 3 ml. portion of the supernatant was recooled to below 5°, mixed with 1 ml. of cooled 1·5 M K_2HPO_4, and kept below 5° for at least 10 minutes but not more than 60 minutes.

While still cool, 3 ml. of the supernatant liquid was then carefully removed from the precipitate of potassium perchlorate and mixed with 0·03 ml. of reduced nicotinamide-adenine dinucleotide solution[1] ($10^{-2}M$) in a 10 mm. cuvette sealed with parafilm. The extinction was measured at 340 mμ with a fixed slit width of 0·2 mm.,

[1]This is now the approved name for diphosphopyridine nucleotide.

using the sensitivity control for the zero extinction reading. The measurement was made against an arbitrary blank of 3 ml. of deionized water containing 0·01 to 0·02 ml. of reduced nicotinamide-adenine dinucleotide.

Exactly 0·03 ml. of lactic dehydrogenase solution[2] (0·75 mg./ml.) was mixed with the test solution by inversion, after sealing the cuvette again with parafilm. Extinction readings against the same blank were recorded after 90 seconds and 120 seconds.

Blank and standard measurements were made in exactly the same way by substituting deionized water, and a standard pyruvic acid solution for blood in the above procedure.

The blank measurement, which was about 0·012, was subtracted from those of standards and unknown. As the reaction between pyruvic acid and reduced nicotinamide adenine dinucleotide is stoichiometric, the pyruvic acid concentration was proportional to the corrected fall in extinction. With the Unicam SP500 spectrophotometer used, an extinction change of 0·100 corresponded to a concentration of 0·60 mg. of pyruvate per 100 ml. of blood. Two corrections, however, were applied. As 3 ml. of blood contained only about 2·54 ml. of water, the volume of solution after deproteinization was 8·54 ml. instead of 9 ml., and therefore the observed result was multiplied by $\frac{8·54}{9}$. Also 3% was added to the observed result to correct as closely as possible for the loss of pyruvate (Huckabee, 1956) in the one-minute interval between withdrawing and deproteinizing the blood. These two corrections were incorporated into the factor for converting extinction values to pyruvate concentration.

OBSERVATIONS ON THE METHOD

The solutions of reduced nicotinamide-adenine dinucleotide and lactic dehydrogenase were obtained in a test kit, but the method described here differs in several ways from that supplied with the reagents. These differences are summarized below, and the reasons for introducing them are discussed.

1 Blood, 3 ml., was deproteinized with 6 ml. instead of 3 ml. of 0·6 M perchloric acid. This was to ensure more complete deproteinization and to provide enough supernatant solution for duplicate analyses. The concentration of the dipotassium hydrogen phosphate solution was raised from 1·1 M to 1·5 M to produce the same *p*H in the buffer system as that which resulted when the smaller volume of perchloric acid was used.

2 The concentrations of reduced nicotinamide-adenine dinucleotide and lactic dehydrogenase in the cuvettes were decreased by 60%, and a blank determination was included with each series of unknowns. These changes were made because of the errors due to the fall in extinction which resulted from dilution of the reduced nicotinamide-adenine

[2]C. F. Boehringer and Soehne, Mannheim, Germany: Pyruvate testkit TC-C.

dinucleotide by the added lactic dehydrogenase, in addition to the fall resulting from its oxidation. This was particularly large using the volume of reagents proposed by the suppliers, especially as the concentration of nicotinamide-adenine dinucleotide was found to be three times as great as that stated. Using the volumes stated above, neglecting to correct for the dilution of nicotinamide-adenine dinucleotide would have caused errors of about 0·07 mg./100 ml. The introduction of a blank assay corrected for the dilution effect but errors could still result from small variations in the volume of enzyme solution added. The reduction in the volume of each of the two reagents used did not impair the ability of the method to measure even quite high levels of pyruvate and produced a corresponding reduction in the errors which could arise from unreproducibility of volume measurement. The reduced nicotinamide-adenine dinucleotide available was equivalent to about 3·5 mg. of pyruvic acid per 100 ml. of blood, and the equilibrium constant of the reaction favoured maximum utilization. Accurate results were obtained on standard solutions corresponding to 3 mg. per 100 ml. of blood. If necessary for the measurement of higher levels, the deproteinized blood can be diluted with 0·4 M perchloric acid before adding phosphate.

3   Volumes were adjusted to provide the 3 ml. of solution required by the 10 mm. cuvettes of the Unicam SP500 spectrophotometer, and an instrument blank was prepared by diluting 0·01 to 0·02 ml. of the nicotinamide-adenine dinucleotide solution in 3 ml. of water. This avoided using the less accurate regions of the extinction scale for the measurement of changes in extinction.

4   A standard was included with each series of unknowns, and in all cases readings were taken 1·5 minutes, as well as two minutes, after adding the enzyme. These precautions were included to check the reagents and confirm that the reaction proceeded at its usual rate. When the correct values for the results of blank and standard assays had been established on numerous occasions, they were then used as a check on the performance of the method subsequently. The extinction readings two minutes after adding lactic dehydrogenase were not more than 0·003 lower than the readings 1·5 minutes after the addition. Larger differences usually indicated contamination of reagents, and the purpose of the 1·5 minute reading was to check that the reaction was by then almost complete. This was less time-consuming than taking several readings at one-minute intervals after two minutes, as others have suggested.

We found that inadequate cooling of the blood during deproteinization with perchloric acid resulted in loss of pyruvic acid, due to persistence of lactic dehydrogenase activity until deproteinization was complete. Once deproteinization was completed, however, the pyruvate in the supernatant solution was stable for at least 24 hours, as demonstrated below. Single analyses were made on each of 17 blood samples. The residual deproteinized solution was left in the centrifuge tube in contact with the precipitated protein, and a second portion was used for analysis the following day. The result on the second day was not significantly different, being an average of 0·01 mg./100 ml. higher than on the first day. The mean difference, ignoring sign, and the standard deviation calculated from these data, were each 0·04 mg./100 ml., similar in fact to the values reported below for duplicates carried out both on the same day.

Errors also resulted if the temperature of the solution was allowed to exceed 5°C during the precipitation of potassium perchlorate, or if the deproteinized blood was not cooled before adding the phosphate. Thus, if the temperature was 8°, 10% of the pyruvate was lost even within the minimum precipitation period of 10 minutes. This was thought to be due to renewed lactic dehydrogenase activity at higher temperatures, after the precipitation of the perchlorate, as the losses occurred with blood samples but not with standard solutions. Provided the temperature of the solution was kept below 5° during precipitation of potassium perchlorate, a delay of 60 minutes did not significantly alter the level of pyruvate.

The precision of the method was investigated by inspecting the results of duplicate analyses. These are reported in Table I for each of three analysts and show a standard deviation for the method of 0·03 mg./100 ml. Single analyses on 13 samples were carried out by two analysts each of whom was unaware of the other's results. The mean result was the same for each analyst, indicating the absence of any systematic difference. Disregarding the sign of each difference, random errors yielded a standard

TABLE I

MEAN DIFFERENCES BETWEEN DUPLICATE RESULTS
AND STANDARD DEVIATIONS FOR REPLICATE RESULTS
CALCULATED FROM THE DIFFERENCES

Analyst	No. of Analyses	Mean Difference	Standard Deviation
1	32	0·03	0·03
2	26	0·04	0·04
3	11	0·02	0·02
2 and 3[1]	13	0·04	0·04
Total	82	0·03	0·03

[1]One of each pair of results was obtained by analyst 2 and the other by analyst 3.

deviation of 0·04 mg./100 ml., no greater than that obtained when one of the analysts made both measurements.

In carrying out recovery experiments, standard pyruvic acid was not added to blood before it had been mixed with perchloric acid, because of the consequent losses which would have occurred. In eight experiments, therefore, pyruvic acid was added to one of two portions of perchloric acid, to each of which portions of blood were then added simultaneously. The amount added corresponded to an increment of 1 mg. per 100 ml. of blood. The mean recovery was 99% and the standard deviation was 4%.

OBSERVATIONS ON THE COLLECTION OF SAMPLES

Venepuncture is facilitated by warming the hand, repeated hand clenching, or venous occlusion. As importance is often attached to the avoidance of artefacts which might influence the pyruvate concentration during the collection of blood, the effect of each of these was investigated in fasting and non-fasting subjects.

In seven experiments the skin temperature of one hand was heated to 5 to 10°C. above the temperature of the other, for at least 30 minutes, and blood was taken simultaneously from the antecubital fossa of each arm. The blood pyruvate concentration for the heated arm was an average of 0·01 mg./100 ml. lower than that for the other (S.D. 0·07 mg./100 ml.) and this difference was not statistically significant $(0·1 < P)$. In only two of the seven experiments was the difference greater than 0·03 mg./100 ml.

In seven other experiments, blood pyruvate concentration was measured before and immediately following a period of 40 to 80 seconds of fist clenching performed at the rate of one clench per second. The pyruvate concentration in the second sample was an average of 0·08 mg./100 ml. higher than in the first (S.D. 0·10 mg./100 ml.). The mean change was not statistically significant $(0·05 < P < 0·1)$ and the degree of change did not appear to be related to the variation in clenching time.

In a further nine experiments the blood pyruvate concentration was measured before and after three minutes of venous occlusion by means of a sphygmomanometer cuff around the upper arm adjusted to a pressure midway between the patient's systolic and diastolic blood pressure. The pyruvate concentration in the second sample was an average of 0·01 mg./100 ml. lower than in the first sample (S.D. 0·11 mg./100 ml.) and this difference was not statistically significant $(0·1 < P)$.

RESULTS

BLOOD PYRUVATE CONCENTRATION IN FASTED SUBJECTS   The pyruvate concentration was estimated in 75 venous blood samples from 30 fasted subjects. The mean level was 0·44 mg./100 ml. (S.D. 0·05) with a range of 0·36 to 0·59 mg./100 ml. In subjects for whom several results were obtained, the variability for the individual was as great as the variability between individuals. The results showed no significant difference between the sexes, the mean value being 0·46 mg./100 ml. for the men, and 0·43 mg./100 ml. for the women. There was no

TABLE II

RESPONSE OF VENOUS BLOOD PYRUVATE AND SUGAR LEVELS TO ORAL GLUCOSE
(1 g./kg. BODY WEIGHT) IN 15 FASTED CONTROLS

Subject	Blood Pyruvate (mg./100 ml.)					Blood Sugar (mg./100 ml.)				
	Minutes after Glucose Administration					Minutes after Glucose Administration				
	0	30	60	90	120	0	30	60	90	120
1	0·37	0·78	0·70	0·83	0·70	75	118	120	126	110
2	0·37	0·87	0·95	0·96	0·73	80	115	140	100	75
3	0·38	0·67	0·96	0·89	0·63	90	115	160	150	110
4	0·40	0·51	1·19	1·01	0·84	70	170	196	130	74
5	0·41	0·42	0·68	0·78	0·73	85	120	156	130	112
6	0·41	0·43	0·62	0·65	0·52	75	135	110	107	75
7	0·42	0·45	0·76	1·26	0·84	90	130	174	150	120
8	0·43	0·54	0·53	0·86	0·75	95	170	196	160	105
9	0·43	0·58	1·06	0·66	0·59	82	120	110	100	80
10	0·44	0·52	0·73	0·86	0·72	70	118	132	115	105
11	0·47	0·53	0·77	0·54	0·52	75	108	135	110	98
12	0·47	0·62	0·73	0·73	0·78	76	110	150	132	118
13	0·47	0·63	0·91	1·00	0·88	82	114	160	150	108
14	0·47	0·82	0·93	0·78	0·53	85	135	180	90	60
15	0·50	0·51	0·97	0·89	0·83	115	155	170	160	112
Mean	0·43	0·59	0·83	0·85	0·71					
S.D.	0·04	0·14	0·19	0·18	0·11					

significant variation of the venous blood levels of pyruvate with age. The mean value for the seven subjects under 25 years of age was the same as for the 11 subjects over 50 years of age, namely, 0·46 mg./100 ml., while the mean blood pyruvate level for the remaining 12 subjects, between 25 and 50 years of age, was 0·42 mg./100 ml.

EFFECT OF ORAL GLUCOSE ON BLOOD PYRUVATE CONCENTRATION   Glucose was given orally to 15 fasted subjects and the venous blood pyruvate and sugar levels were measured at 30-minute intervals for two hours. The results are reported in Table II. In all cases the oral glucose tolerance curve was within normal limits.

The mean blood pyruvate curve for these 15 subjects is shown in Fig. 1. From a mean fasting level of 0·43 mg./100 ml. the mean pyruvate level rose by nearly 100% to 0·85 mg./100 ml. and then fell to 0·71 mg./100 ml. two hours after glucose administration. The highest level of blood pyruvate found was 60 minutes after glucose administration in six subjects, after 90 minutes in eight subjects, and after 120 minutes in the remaining subject.

EFFECT OF INTRAVENOUS GLUCOSE ON BLOOD PYRUVATE CONCENTRATION   Glucose was given to 15 fasted subjects by intravenous injection. Venous blood samples were taken at 30-minute intervals for two hours for the measurement of pyruvate and at 15-minute intervals for two hours for the measurement of sugar. The results reported in Table III show that intravenous glucose tolerance was normal in all cases.

The mean blood pyruvate curve for these 15 subjects is shown in Fig. 2. From a fasting level of 0·43 mg./100 ml. the mean pyruvate level rose by nearly 100% to 0·82 mg./100 ml. and then fell to 0·50 mg./100 ml. two hours after glucose administration. The highest level of blood pyruvate was found 30 minutes after glucose administration in eight subjects and after 60 minutes in the remaining seven subjects.

DISCUSSION

The measurement of pyruvate as its hydrazone gives higher values than those obtained using specific methods, as the hydrazones of other α-keto acids are also partially measured (Bauer, 1956). Thus, using a hydrazone method, Bueding and Wortis (1940) found a mean blood pyruvate level of 0·98 mg./100 ml. (S.D. 0·09) in 60 fasted subjects compared with 0·44 mg./100 ml. (S.D. 0·05, range 0·36 to 0·59) found in the present study. Using the nicotinamide-adenine dinucleotide reaction for the specific measurement of blood pyruvate in normal fasted subjects, the mean value obtained by Bauer (1956) was 0·53 mg./100 ml. (range 0·30 to 0·81), by Redetzki *et al.* (1956) 0·57 mg./100 ml. (S.D. 0·16), by Segal *et al.* (1956) 0·66 mg./100 ml. (range 0·39 to 0·86), and by Tsirimbas and Stich (1960) 0·56 mg./100 ml. (S.D. 0·11). These results are higher than those we obtained, possibly because the other workers did not subtract a blank reading to allow for the fall in reduced nicotinamide-adenine dinucleotide extinction due to dilution by lactic dehydrogenase. The range and standard deviation obtained by these workers are also larger than in the present study.
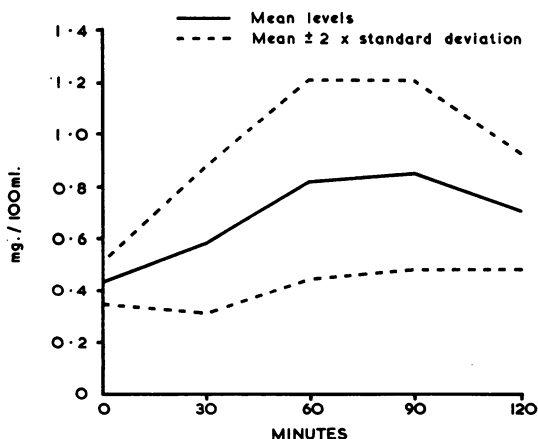

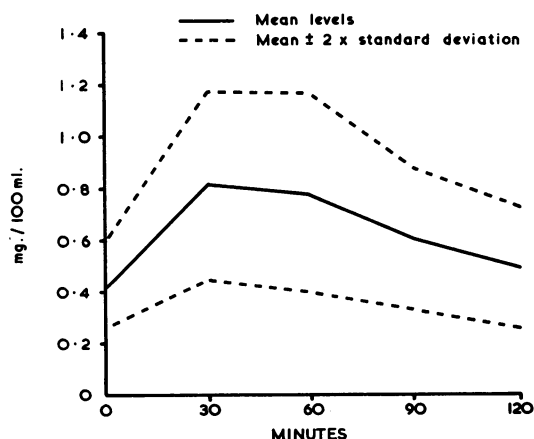FIG. 1.   *Blood pyruvate levels after the oral administration of glucose.*

FIG. 2.   *Blood pyruvate levels after the intravenous administration of glucose.*

TABLE III

RESPONSE OF VENOUS BLOOD PYRUVATE AND SUGAR LEVELS TO INTRAVENOUS GLUCOSE
(0·5 g./kg. BODY WEIGHT) IN 15 FASTED CONTROLS

Subject	Blood Pyruvate (mg./100 ml.)					Blood Sugar (mg./100 ml.)							
	Minutes after Glucose Administration					Minutes after Glucose Administration							
	0	30	60	90	120	0	30	45	60	75	90	105	120
1	0·35	0·66	0·83	0·54	0·29	74	186	160	139	115	104	85	70
2	0·35	0·95	1·13	0·79	0·54	75	176	130	100	80	75	60	65
3	0·36	0·58	0·70	0·64	0·60	80	195	158	120	100	80	76	70
4	0·38	0·87	0·95	0·86	0·73	85	249	211	160	130	110	96	90
5	0·39	0·81	0·39	0·35	0·33	85	245	197	158	130	100	90	80
6	0·39	0·90	0·82	0·65	0·61	95	192	158	128	113	100	70	75
7	0·42	0·62	0·72	0·63	0·38	76	204	153	118	98	80	—	—
8	0·42	0·94	0·84	0·51	0·53	71	201	153	128	108	81	—	60
9	0·43	0·62	0·65	0·56	0·52	70	255	202	154	129	114	—	96
10	0·44	0·62	0·63	0·57	0·41	75	195	149	114	81	80	80	75
11	0·44	1·13	1·04	0·58	0·40	80	200	160	132	118	95	90	80
12	0·46	0·93	0·77	0·62	0·50	80	210	153	121	104	80	72	70
13	0·49	0·83	0·73	0·48	0·44	85	188	149	110	88	75	70	66
14	0·53	1·11	1·02	0·87	0·77	85	200	160	124	96	80	80	76
15	0·65	0·84	0·57	0·52	0·47	75	168	129	98	90	70	—	75
Mean	0·43	0·82	0·79	0·61	0·50								
S.D.	0·08	0·18	0·19	0·14	0·12								

Several workers (Bueding, Wortis, and Fein, 1942; Klein, 1942; Bueding and Goldfarb, 1943; Joiner, McArdle, and Thompson, 1950; Shrifter and Kritzer, 1956) found a transient rise in the blood pyruvate concentration after glucose loading, and found abnormalities of the pyruvate curve after glucose in several diseases, for example, diabetes mellitus and thiamine deficiency. These workers used non-specific hydrazone methods for the measurement of the blood pyruvate. The pyruvate levels after glucose have also been measured by a more specific chromatographic modification (Thompson, Butterfield, and Fry, 1960; McArdle, Mackenzie, and Webster, 1960).

We are not aware of any report on the changes in blood pyruvate concentration, measured by the specific reduced nicotinamide-adenine dinucleotide reaction, after the oral and intravenous administration of glucose to control subjects. The results presented here will be used in the interpretation of results, to be reported later, obtained from patients with conditions which may affect pyruvate metabolism.

We thank Miss G. Robertson who carried out many of the analyses and Mr. H. D. Philps who kindly translated the relevant German literature. One of us (J.L.) gratefully

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