Indirect Analysis of Corticosteroids

5. THE DETERMINATION OF 17-DEOXYCORTICOSTEROIDS

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Naturally occurring 17-deoxycorticosteroids (the term is used here to denote corticosteroids unsubstituted at position 17) comprise 21-hydroxy-pregnan-20-ones (I) and pregnane-20:21-diols (II). Oxidation with sodium bismuthate ruptures their 20-21 bond with the resultant formation of form-

aldehyde from (I) and (II), of aetiocolanic acids C-20 aldehydes and the measurement of the latter.

(III) from (I) and of 21-norpregnan-20-als (IV) from (II) (Brooks & Norymberski, 1953). The last conversion was presumed; it is now established by the isolation of 3 β -hydroxy-21-norpregn-5-en-20-al (VI) (as its acetate) from the reaction of pregn-5-ene-3 β :20 β :21-triol (V) with sodium bismuthate. Reduction with borohydride followed by oxidation with sodium bismuthate was expected to convert 20:21-ketols (I) into C-20 aldehydes (IV) as well. In

The Angeli-Rimini reaction (Angeli, 1892; Rimini, 1901) was chosen for the detection of aldehydes because of its specificity: only aldehydes are known to react and only those which lack substituents interacting with the aldehyde group. The reaction is usually performed according to Rimini by treating aldehydes (VII) with benzenesulphohydroxamic acid in a weakly alkaline solution. The hydroxamic acids (VIII) formed are then detected in the reaction mixture by conversion into their purple ferric complexes (IX).

* Part 4: Norymberski & Stubbs (1956).

Preliminary experiments

The conditions outlined above were found unsuited to the accurate determination of small amounts ($< 1 \mu\text{mole}$) of aldehydes because of large and variable reagent blanks presumably due to surplus benzenesulphohydroxamic acid and to the formed benzenesulphonic acid (Struck, 1956). However, when the formed hydroxamic acid was extracted with ethyl acetate, the interfering substances remained in the aqueous phase. The ferric hydroxamate was formed by shaking the extract with a solution of ferric nitrate in perchloric acid. The complex was then found in the organic phase but was quantitatively transferred into the aqueous phase on addition of light petroleum; by suitably adjusting the volumes of extract, reagent and diluent almost a five-fold concentration of the complex was achieved. Its spectrum in either phase showed the typical absorption maximum at $510 \text{ m}\mu$ (Fig. 1). Either procedure was found satisfactory for the determination of pure aldehydes but only the latter, involving the preparation of an aqueous concentrate, was applicable to the intended determination of 17-deoxycorticosteroids in the urine. Therefore, what follows refers to results obtained by the latter technique, with 3-oxobisnorchol-4-en-22-al as compound of reference. In terms of this compound ($= 100$), 3β -acetoxy-norpregn-5-en-20-al (VI-acetate) was found to have a colour equivalent of 92 (mole/mole). Colorimetric measurements were found to obey Beer's law (Fig. 2, curve A). Their reproducibility was improved by the use of Allen's correction (Allen, 1950). Agreement between duplicate determinations carried out concurrently was good (coefficient of

variation 3%, calculated from 26 measurements); agreement between determinations carried out on different days was less satisfactory (coefficient of variation 8%, calculated from 32 measurements). It is therefore desirable that all determinations be expressed in terms of a model compound assayed concurrently with the analytical sample under investigation.

Three 20:21-ketols and one 20:21-glycol (compounds 1, 2, 3 and 6 in Table 1) were treated consecutively with potassium borohydride and sodium bismuthate; by the analytical test outlined, all four compounds gave aldehydes in high yield (Table 1, column A). The formed ferric hydroxamates exhibited absorption spectra identical with those derived from pure aldehydes. Submitted to the same sequence of reactions, aldosterone and cholestane- $3\beta:5\alpha:6\beta$ -triol failed to yield ferric hydroxamates, presumably owing to interaction between the formed aldehyde group and a neighbouring oxygen function. Four compounds of the androstane series and one of the oestrane series with vicinal oxygen functions at positions 16 and 17 gave ferric hydroxamates in poor yield (Table 1), interaction presumably occurring between the two aldehyde groups of the expected 16:17-secodialdehydes.

The proposed determination of 17-deoxycorticosteroids in the urine was facilitated by their stability towards hot mineral acid (Reichstein & Shoppee, 1943; Tompsett, 1953; see also Table 1, column B) since it permitted the determination to be performed on the readily prepared extracts of acid-treated urines. Preliminary experiments showed that such extracts, when analysed for 17-deoxycorticosteroids, gave rise to asymmetrical absorption spectra, indicating the presence of material absorbing non-specifically in the spectral region 400–600 $\text{m}\mu$. In order to ascertain the spectroscopic properties of this material, determinations were carried out on 'urinary blanks' obtained by omitting the addition of ferric ion at the final stage of the assay. The blanks exhibited approximately linear absorption

Fig. 1. Spectra of the ferric hydroxamate from 3-oxobisnorchol-4-en-20-al: ●, $0.5 \mu\text{mole}$ in ethyl acetate extract; ○, $0.1 \mu\text{mole}$ in aqueous concentrate; ▲, reagent blank in ethyl acetate; □, reagent blank in aqueous concentrate. ● and ○ have been corrected by subtraction of corresponding reagent blanks.

Fig. 2. Proportionality in the determination of aldehydes and of 17-deoxycorticosteroids. ●, 3-Oxobisnorchol-4-en-22-al; ■, $3\alpha:21$ -dihydroxy- 5β -pregnane-11:20-dione, added to urine.

Table 1. *Determination of ketols and glycols*

A: Direct determination; *B*: determination after treatment with hot HCl; *C*: compound added to urine and determined by standard procedure. Results are in terms of 3-oxobisnorchol-4-en-22-al (mole/mole); mean values are given, with their s.d.; figures in parentheses denote the number of determinations.

	Compound	Procedure			Quantity assayed (μ g.)
		<i>A</i> (% found)	<i>B</i> (% found)	<i>C</i> (% found)	
1	11-Deoxycorticosterone	94 $\pm$ 2 (25)	87 $\pm$ 2 (3)	82 $\pm$ 2 (2)	20-250
2	Corticosterone	93 $\pm$ 7 (8)	86 $\pm$ 4 (8)	103 $\pm$ 6 (5)	20-50
3	3 α :21-Dihydroxy-5 β -pregnane-11:20-dione	99 $\pm$ 2 (7)	—	96 $\pm$ 7 (12)	10-100
4	3 α :21-Diacetoxy-5 β -pregnane-11:20-dione	—	—	105 $\pm$ 4 (3)	50
5	5 β -Pregnane-3 α :11 β :20 β :21-tetrol	—	82 $\pm$ 2 (2)	83 $\pm$ 2 (2)	40-45
6	Pregn-5-ene-3 β :20 β :21-triol	90 $\pm$ 3 (2)	—	87 $\pm$ 2 (4)	50
7	Aldosterone	0 (1)	—	—	12-5
8	3 α :17 α :21-Trihydroxy-5 β -pregnane-11:20-dione	0.6 $\pm$ 0 (3)	—	0.0 $\pm$ 0 (3)	360-500
9	Cholestane-3 β :5 α :6 β -triol	0.0 $\pm$ 0 (2)	—	—	22
10	3 β :16 α -Dihydroxyandrost-5-en-17-one	18.0 $\pm$ 0 (2)	—	—	40
11	3 β :17 β -Dihydroxyandrost-5-en-16-one	23.3 $\pm$ 3 (4)	—	—	40-170
12	Androst-5-ene-3 β :16 α :17 β -triol	19.0 $\pm$ 0 (2)	—	—	41
13	Androst-5-ene-3 β :16 β :17 β -triol	18.0 $\pm$ 0 (2)	—	—	40
14	Oestriol methyl ether	23.0 $\pm$ 0 (2)	—	—	25

whilst analytical samples treated in the usual manner, when corrected by subtraction of the blanks, gave the typical spectra of ferric hydroxamates (Fig. 3). It follows that the determination of 'urinary blanks' is unnecessary since their colorimetric contribution can be eliminated by the application of Allen's correction. Parallel determinations of samples of a urine treated with acid and with β -glucuronidase respectively gave practically identical results (see Table 2); hence, there is no reason to suspect that treatment with acid leads to the formation of artifacts interfering with the assay. The standard procedure was found accurate (coefficient of variation between duplicate determinations 4%, calculated from 67 measurements), it accounted satisfactorily for varying amounts of pure 17-deoxycorticosteroids added to the urine (Table 1, column *C*; Fig. 2, curve *B*), and its results were not affected by the presence of relatively large quantities of 3 α :17:21-trihydroxy-5 β -pregnane-11:20-dione (tetrahydrocortisone) (Table 1). Average excretion of 17-deoxycorticosteroids by 16 healthy men was 3.4 mg./24 hr. (range 2.3-5.2; s.d. 0.8) and by eight healthy women it was 3.1 mg./24 hr. (range 2.0-4.7; s.d. 1.0).

Since oxidation with sodium bismuthate of 21:21-ketols (I) yields aetiocholic acids (III) and that of 20:21-glycols (II) C-20 aldehydes (IV), it is clear that omission of the reduction step from the standard procedure provides a method for the determination of the latter subgroup (II) of 17-deoxycorticosteroids. 20:21-Glycols were so determined in urines from 12 normal subjects and were found to account on the average for 71% (range 53-80; s.d. 8) of the total 17-deoxycorticosteroids (see Table 3).

Brief reference to this work was made previously (Norymberski, 1961).

MATERIALS AND METHODS

Preparation of 3 β -acetoxy-21-norpregn-5-en-20-al (VI-acetate)

Pregn-5-ene-3 β :20 β :21-triol (V) (100 mg.) in aqueous acetic acid (100 ml.; 50%, v/v) was shaken for 5 min. with

Table 2. *Comparison of two hydrolytic procedures in the assay of urinary 17-deoxycorticosteroids*

Subjects 1-3 were untreated obese women. Subject 4 was an obese man treated with corticotrophin.

Subject no.	17-Deoxycorticosteroids (mg./24 hr.)	
	Hydrolysis by acid	Hydrolysis by enzyme
1	4.5	4.0
2	4.7	4.55
3	4.4	3.9
4	11.5	12.5

Table 3. *20:21-Glycols and 20:21-ketols in normal human urine*

For details see text.

Subject no.	17-Deoxycorticosteroids (mg./24 hr.)	20:21-Glycols (mg./24 hr.)	20:21-Ketols (mg./24 hr.)
1	2.9	2.1	0.8
2	4.1	3.2	0.9
3	3.2	2.5	0.7
4	3.4	2.4	1.0
5	2.5	1.8	0.7
6	5.2	3.6	1.6
7	2.7	1.9	0.8
8	3.3	2.6	0.7
9	1.9	1.4	0.5
10	4.7	2.8	1.9
11	3.6	2.8	0.8
12	4.4	2.3	2.1
Mean	3.5	2.5	1.0
s.d.	1.0	0.6	0.5

sodium bismuthate (10 g.). Surplus reagent was filtered off, washed with a little methanol, the filtrate was diluted with water (100 ml.) and extracted with ethyl acetate. The

Fig. 3. Spectra obtained in the assay of six normal urines (curves A-F) and the corresponding 'urinary blanks' (curves a-f). All readings are corrected as described in the text.

crude product was treated with acetic anhydride in pyridine overnight at room temperature. The pale-yellow product was decolorized with charcoal in hexane, whereby colourless crystals of (VI)-acetate (95 mg.) were obtained; m.p. 125–140° (Kofler stage). Material recrystallized from hexane had m.p. 130–140° (Kofler stage), 145–152° (capillary), 156–157° (evacuated capillary); $[\alpha]_D^{20} -20 \pm 2^\circ$ in CHCl_3 (c. 1.57); ν_{max} in CS_2 for CHO 2700 and 1720 (shoulder) cm^{-1} , for $\text{CH}_3\cdot\text{CO}_2$ 1730 and 1240 cm^{-1} (Found: C, 76.2; H, 9.6. Calc. for $\text{C}_{22}\text{H}_{32}\text{O}_5$: C, 76.7; H, 9.4%). Miescher, Hunziger & Wettstein (1940) reported m.p. 169–171° $[\alpha]_D^{20} -13.5 \pm 4^\circ$.

Analytical

All solvents but methylene dichloride and ethylene dichloride were of analytical grade; all but light petroleum (b.p. 60–80°) were distilled. Colorimetric measurements were carried out with a Hilger Uvispek spectrophotometer in microcells of 1 cm. light-path and 0.2 ml. capacity.

(i) *Determination of aldehydes.* The analytical sample, containing 5–50 μg . of aldehyde, is dissolved in *tert.*-butanol (0.2 ml.), treated with a freshly prepared solution of benzenesulphohydroxamic acid in *tert.*-butanol (0.1 ml.; 0.1 M) and with a solution (0.1 ml.) of sodium hydroxide (0.125 N) in aq. *tert.*-butanol (50%, v/v). After 30 min. at

room temperature, water (0.5 ml.) is added and after a further 15 min. the mixture is shaken with ethyl acetate (1.0 ml.) for 15 min. and then centrifuged at 4000 rev./min. for 5 min. A portion of the top layer (1.0 ml.) is carefully removed and shaken for 5 min. with a solution (0.1 ml.) of ferric nitrate (0.5N) in perchloric acid (2.5N); light petroleum (2.0 ml.; b.p. 60–80°) is added and the mixture shaken again. It is then centrifuged, the organic phase carefully removed and the aqueous phase washed with a mixture of ethyl acetate and light petroleum (3.0 ml.; 1:2, v/v). The extinction of the aqueous layer is determined at 440, 510 and 580 m μ against a process blank obtained by submitting *tert.*-butanol (0.2 ml.) alone to the treatment. The readings are evaluated according to Allen (1950) and expressed in terms of a pure standard aldehyde: here, 3-oxobisnorchol-4-en-22-al.

Pure aldehydes and those prepared from pure 17-deoxycorticosteroids (see below) can be determined in the ethyl acetate extract after its treatment with the ferric reagent. When a blank is required, accounting for coloured impurities ('urinary blank' with processed urine extracts), the procedure is varied as follows. The volumes of solvents and solutions employed are doubled. From the ethyl acetate extract two equal samples (1.0 ml. each) are taken; one sample (*A*) is treated with the ferric reagent, the other (*B*) with perchloric acid only. All following operations are performed as described above. Preparation of the process blank is modified in the same manner (samples *a* and *b*). The extinction of the ferric hydroxamate is found as $(A - B) - (a - b)$.

(ii) *Determination of 17-deoxycorticosteroids.* The analytical sample, containing 10–100 μ g. of 17-deoxycorticosteroids, is dissolved in *tert.*-butanol (0.2 ml.) and treated with a freshly prepared aqueous solution of potassium borohydride (0.1 ml.; 20%, w/v) overnight at room temperature. Aqueous acetic acid (3 ml.; 40%, v/v) and sodium bismuthate (0.5 g.) are added, the mixture shaken for 10 min., centrifuged, a portion of the supernatant (2.5 ml.) treated with aq. sodium metabisulphite (0.05 ml.; 20%, w/v) and with hydrochloric acid (1 ml.; 5N) and then shaken with methylene dichloride (3.0 ml.) for 15 min. The aqueous phase is removed and the extract (i.e. lower phase) washed successively with water (1 ml.), 3N-NaOH (1 ml.; shaken for 5 min.) and water (2 $\times$ 1 ml.). The washed extract is filtered through anhydrous sodium sulphate and a portion (2.0 ml.) evaporated to dryness, care being taken that the residue is not overheated. The residue is treated as described under (i).

Selective determination of 20:21-glycols is carried out by omitting treatment with potassium borohydride but otherwise in exactly the same manner as above.

The time of oxidation with sodium bismuthate was varied between 5 and 15 min. with satisfactory results. Shorter and longer reaction times lead to lower yields of aldehydes.

(iii) *Determination of 17-deoxycorticosteroids in the urine.*

(a) With acid hydrolysis (standard procedure). A 24 hr. urine specimen is made up to 2.0 l. with water. A sample (60.0 ml.) of the diluted urine with conc. hydrochloric acid (20.0 ml.) in a glass-stoppered tube (approx. capacity 120 ml.) is heated for 10 min. in a boiling-water bath and then rapidly cooled. The mixture is shaken with ethylene dichloride (20.0 ml.) for 15 min., most of the aqueous phase removed with suction and the extract transferred into a glass-stoppered centrifuge tube (approx. capacity

40 ml.). Any emulsion is broken down by centrifuging, the remaining aqueous phase removed and the extract washed successively with water (4 ml.), 3N-NaOH (4 ml.; shaken for 5 min.) and water (2 $\times$ 4 ml.). The washed extract is filtered through a layer of anhydrous sodium sulphate and a portion (15.0 ml.) evaporated to dryness. The residue is treated as under (ii).

(b) With enzymic hydrolysis. One urine specimen was treated with bacterial β -glucuronidase (Sigma Chemical Co.) (300 Sigma units/ml., pH 6.8, 36 hr. at 37°), three other specimens with bovine β -glucuronidase (Ketodase, Warner-Chilcott) (450 Fishman units/ml., pH 4.8, 60 hr. at 37°; see Talalay, Fishman & Huggins, 1946). Each urine was then extracted with chloroform (3 $\times$ 0.5 vol.), the extract washed successively with 3N-NaOH (0.2 vol.) and water (3 $\times$ 0.2 vol.), dried with anhydrous sodium sulphate and evaporated to dryness *in vacuo*. Suitable portions of this material were assayed as described under (ii) and the results compared with those obtained by method (a) (see Table 2).

DISCUSSION

An earlier attempt at the group determination of urinary 17-deoxycorticosteroids (Tompsett, 1953), as acid-resistant formaldehydogenic substances, proved unsuccessful when it was shown that cortisone and Reichstein's substance S (and presumably also other 17:21-diol-20-ones) significantly contribute to the assay (Marrian, Paterson & Atherden, 1953). The α -ketolic subgroup of 17-deoxycorticosteroids (20:21-ketols) has been determined as reducing substances non-chromogenic in the Porter-Silber reaction (Romani, Bugard & Fischer, 1956) and as reducing substances present in a chromatographic zone considered free of other reducing material (Richardson *et al.* 1955). Currently, the analytical method of choice is the determination of individual compounds of the group after their chromatographic separation. So far, 12 such compounds (i–xii, Table 4) have been found in human urine. They were originally isolated from the urine of subjects treated with corticotrophin, corticosterone, 11-dehydrocorticosterone or 11-deoxycorticosterone (Table 4); seven of them (i–vii), all 20:21-ketols, were later found also in the urine of untreated normal subjects (Romani, 1956*a, b*; Romani & Ableaux-Fernet, 1956).

The present method is considered specifically to determine neutral acid-resistant α -glycols and α -ketols which on consecutive treatment with borohydride and with sodium bismuthate yield non-volatile aldehydes. Among steroids known to occur in the human urine these requirements are met by 17-deoxycorticosteroids and by the 16:17-glycols of the androstane series. Since the latter group of compounds is excreted by man in relatively small amounts (Fotherby, Colas, Atherden & Marrian, 1957) and since they are converted into ferric hydroxamates in very poor yield (see Table 1) it is considered that their contribution to the assay

Table 4. 17-Deoxycorticosteroids in human urine

Compound	Reference
(i) 21-Hydroxypregn-4-ene-3:20-dione (11-Deoxycorticosterone)	Richardson <i>et al.</i> (1955)
(ii) 3 α :21-Dihydroxy-5 β -pregnan-20-one	Richardson <i>et al.</i> (1955)
(iii) 21-Hydroxypregn-4-ene-3:11:20-trione (11-Dehydrocorticosterone, substance A)	Touchstone, Bulaschenko & Dohan (1955)
(iv) 11 β :21-Dihydroxypregn-4-ene-3:20-dione (Corticosterone, substance B)	Touchstone, Bulaschenko, Richardson & Dohan (1954)
(v) 3 α :11 β :21-Trihydroxy-5 β -pregnan-20-one	Touchstone <i>et al.</i> (1954)
(vi) 3 α :11 β :21-Trihydroxy-5 α -pregnan-20-one	Touchstone <i>et al.</i> (1954); Engel, Carter & Springer (1954)
(vii) 3 α :21-Dihydroxy-5 β -pregnane-11:20-dione	Touchstone <i>et al.</i> (1954); Engel <i>et al.</i> (1954)
(viii) 3 α :21-Dihydroxy-5 α -pregnane-11:20-dione	Peterson (1959)
(ix) 3 β :21-Dihydroxypregn-5-en-20-one	Dobriner & Lieberman (1952)
(x) 11 β :20 β :21-Trihydroxypregn-4-en-3-one	Richardson, Bulaschenko & Dohan (1958)
(xi) 11 β :20 α :21-Trihydroxypregn-4-en-3-one	Bulaschenko, Richardson & Dohan (1960)
(xii) 20 β :21-Dihydroxypregn-4-ene-11:20-dione	Bulaschenko <i>et al.</i> (1960)

is negligible. The 16:17-ketols being unstable to acid (Marrian, Watson & Panattoni, 1957) need not be here considered. The excellent correlation found between the excretion of 17-hydroxycorticosteroids and of 17-deoxycorticosteroids by normal untreated subjects and by subjects whose adrenal function was suppressed by treatment with 9 α -fluorocortisol (Norymberski, 1961) indicates adrenal origin of the compounds determined by the present method. Assuming that the assay is specific, its elaboration to the separate determination of 20:21-glycols and 20:21-ketols reveals the presence of considerable quantities of the former in the urine of healthy men and women. Although three 20:21-glycols containing the 4-en-3-one system (x-xii) were found in small quantities in the urines of patients treated with corticosterone or with the acetate of 11-dehydrocorticosterone (Richardson *et al.* 1958; Bulaschenko, Richardson & Dohan, 1960) it is most unlikely that these compounds account for more than a small fraction of the 20:21-glycols detected by the group assay in the urine of normal subjects. It is therefore suggested that man excretes substantial amounts of as yet unidentified 20:21-glycols.

SUMMARY

1. The Angeli-Rimini reaction was adapted to the determination of small quantities ($\geq 10 \mu\text{m}$ -moles) of aldehydes.

2. A method was developed for the group determination of 17-deoxycorticosteroids by their conversion into 21-norpregnan-20-als and the measurement of the latter by means of the modified Angeli-Rimini reaction. The method was successfully applied to the determination of 17-deoxycorticosteroids in extracts of acid-treated urines.

3. The above method was modified to permit the separate determination of 21-hydroxypregnan-20-ones and of pregnane-20:21-diols. Applied to urines of normal subjects, the differential assay revealed the presence of substantial quantities of as yet unidentified compounds of the 20:21-diol group.

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The Intracellular Distribution, Latency and Electrophoretic Mobility of L-Glutamate-Oxaloacetate Transaminase from Rat Liver

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Previous studies of the distribution of L-glutamate-oxaloacetate transaminase in subcellular fractions, obtained by differential centrifuging of liver homogenates, have all shown that this enzyme is present in both supernatant and mitochondria, with a somewhat higher percentage of total activity in the supernatant fraction (Müller & Leuthardt, 1950; Asada, 1958; Gaull & Vilee, 1960). There are no previous reports of investigations of the latency and activation of L-glutamate-oxaloacetate transaminase in mitochondria, although an increase of activity in liver homogenates during storage has been reported (Schmidt, Schmidt & Wildhirt, 1958) and aging is known to activate latent mitochondrial enzymes. Green, Leloir & Nocito (1945) reported that purified L-glutamate-oxaloacetate transaminase could be separated by electrophoresis into three components and that activity was associated with two of these. It has recently been shown (Fleisher, Potter, Wakim, Pankow & Osborne, 1960) that two L-glutamate-oxaloacetate transaminases with different electrophoretic mobilities have different substrate affinities and pH-dependence. The following experiments show a relationship between the electrophoretic mobility of the enzyme and its localization within the cell.

EXPERIMENTAL

Enzyme-assay procedures

Several procedures were used to assay L-glutamate-oxaloacetate transaminase. Units of transaminase activity were defined as μ moles of oxaloacetate or L-glutamate formed/hr. at 25°.

L-Glutamate-oxaloacetate-transaminase assay by the method of Reitman & Frankel (1957). The amount of oxaloacetate formed during incubation for 1 hr. at 37° was measured colorimetrically with 2:4-dinitrophenylhydrazine. The results were converted into μ moles of oxaloacetate formed/hr. at 25° (King, 1960).

L-Glutamate-oxaloacetate-transaminase assay by the method of Karmen (1955). In this method the rate of formation of oxaloacetate is determined, reduced diphosphopyridine nucleotide (DPNH) and malic dehydrogenase being added to the assay system so that DPNH is oxidized as oxaloacetate is produced. The resulting decrease in extinction at 340 m μ was followed in a Unicam SP. 500 spectrophotometer; the progress curve was linear. A fall in *E* of 0.01 is equivalent to the formation of 4.83 μ m-moles of oxaloacetate in the reaction mixture. The temperature was maintained at 25° by use of a cell housing through which water from a thermostat was circulated. A higher concentration of L-aspartate, 66.6 mM, was used than that in the original method (33.3 mM). The concentration of α -oxoglutarate was 6.7 mM.

It was found that in the presence of NH_4^+ ions some glutamic-dehydrogenase activity is measured in the Karmen method. The commercial malic dehydrogenase added in this test contains $(\text{NH}_4)_2\text{SO}_4$, 9 mM final concentration. To correct for this the oxidation of DPNH was followed in the presence of all reactants except L-aspartate and this control rate was subtracted from the final rate after adding aspartate, allowance being made for dilution. A correction of up to 10% was sometimes necessary. A slight inhibition of transamination, 2–5%, due to these NH_4^+ ions, was ignored.

L-Glutamate-oxaloacetate-transaminase assay by the method of Cammarata & Cohen (1951). The rate of oxaloacetate production is measured spectrophotometrically at 280 m μ . Preliminary experiments showed that the substrate concentrations in the original descriptions by Cammarata & Cohen (1951) were suboptimum for mitochondrial preparations; hence they were both increased from the