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MITOCHONDRIA FROM HUMAN TERM PLACENTA

I. ISOLATION AND ASSAY CONDITIONS FOR OXIDATIVE PHOSPHORYLATION

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SUMMARY

Human term placental mitochondria were resolved by differential centrifugation into three fractions, heavy mitochondria, light mitochondria and a third, less dense fraction. Approximately equal amounts of mitochondrial protein were found in the three fractions.

These mitochondrial preparations differed in physical properties, ATPase and "ADPase" content and oxidative capacities.

Assay conditions were developed which permitted the polarographic measurement of respiration and coupled phosphorylation carried out by all three mitocondrial preparations despite the variable nucleotide-phosphate phosphatase activities present. With heavy mitochondria, rates of respiration were consistently higher than those previously reported for unfractionated placental mitochondria. Respiratory control ratios were comparable to those of mitochondria from other steroid hormone-producing endocrine tissues and ADP/O ratios approaching the theoretical maxima were obtained.

Both lighter placental mitochondrial fractions displayed somewhat lower respiration rates and respiratory control but their primary defect was a selective uncoupling of the third site of energy conservation.

Modification of isolation procedures were evaluated in terms of quantitative yield and functional activity of the three fractions.

INTRODUCTION

In early studies [1, 2] of the oxidative phosphorylating capacity of human term placental mitochondria, great variability and instability were encountered. Low rates of respiration were obtained and attempts to assay coupled ATP production by means of inorganic phosphate uptake were hampered by the presence of enzymes catalyzing the release of phosphate from both ATP and ADP. Low and variable P/O ratios were observed using NAD-linked substrates but it was suggested that this

might not necessarily be indicative of an uncoupled state of respiration in these mitochondria. More recently, Klimek et al. [3] were able to improve the P/O ratios of placental mitochondria by the addition of bovine serum albumin to homogenates prior to the isolation of mitochondria; but no significant enhancement of respiration was obtained.

Our approach in this study to obtain more active and reproducible mitochondrial preparations was to fractionate the total yield of mitochondria in order to remove inactive, damaged or contaminating particles and to design incubation conditions which would minimize the influence of interfering phosphatase activities upon assays of oxidative phosphorylation.

MATERIALS AND METHODS

Sucrose (grade I), Tris buffer (Trizma base and Trizma HCl, reagent grade), bovine serum albumin (essentially fatty acid free), L-glutamic acid, α-ketoglutaric acid, 2, 4-dinitrophenol (grade II) and xanthine oxidase (type C-300) were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A. ATP and ADP were obtained from P-L Biochemicals, Milwaukee, Wisc., U.S.A. EDTA and *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD) were products of the Eastman Kodak Co., Rochester, N.Y., U.S.A. Malic acid and the sodium salts of succinic acid, DL-isocitric acid (*allo*-free), and DL-β-hydroxybutyric acid were purchased from Calbiochem, San Diego, Calif., U.S.A. Sodium pyruvate was obtained from Matheson Coleman and Bell, East Rutherford, N.J., U.S.A. and L-ascorbic acid from Mallinckrodt Chemical Works, St. Louis, Mo., U.S.A. All other chemicals were of reagent grade quality. Water used for all solutions was doubly distilled in an all-quartz apparatus.

Tissue preparation – isolation of mitochondria

Human term placentas were processed at 0–4 °C within 60 min of delivery. The blood was drained, membranes removed and the tissue passed through a meat grinder. The resulting mince was washed (3 times) by suspending in 3 volumes of Washing Medium (0.25 M sucrose, 0.154 M KCl, 1 mM EDTA, adjusted to pH 7.4 with 1 M KOH) and filtered through 2 layers of surgical gauze. The washed mince was blotted on absorbent paper. Approximately 100–120 g of washed mince were suspended to 450 ml in Isolation Medium (0.25 M sucrose, 1 mM EDTA, pH adjusted to 7.4 with 1 M KOH introducing 1 mM K^+) and was homogenized by nine strokes in a glass homogenizer with a tightly fitted Teflon pestle driven at 1200 rev./min. The homogenate was centrifuged at $750 \times g$ for 10 min and the pellet discarded. The mitochondrial fraction was sedimented at $10\,800 \times g$ for 10 min.

Mitochondrial fractionation

The total mitochondrial preparation isolated in the above procedure was fractionated following the procedure of Green and Ziegler [4]. Mitochondria were suspended in 20 vols of Resolving Medium (0.25 M sucrose, 1 mM EDTA, 1 mM Tris · HCl, pH adjusted to 7.8 just prior to use) and centrifuged for 10 min at $12\,000 \times g$. A two-layered pellet composed of tightly adhering heavy mitochondria (fraction I) and loosely packed light mitochondria (fraction II) was obtained and, in

addition, a cloudy supernatant fraction (S_1), which was decanted and retained. Light mitochondria were differentially dislodged from the two-layered pellet and suspended in Isolation Medium as described by Green and Ziegler and were washed three times by centrifugation at 10 500 × *g* for 10 min after suspension in 15 vols of this medium. Heavy mitochondria which separated during the washing of light mitochondria were retained and combined with the original pellet of heavy mitochondria. This combined heavy fraction was washed once by suspension in 20 vols of Isolation Medium and centrifugation at 12 000 × *g* for 10 min. The three supernatant fractions obtained from the washing of the light mitochondria were combined with S_1 and an additional mitochondrial fraction (fraction III) was obtained by centrifugation at 15 000 × *g* for 10 min. This pellet was washed by resuspension in 20 vols of Isolation Medium and centrifugation at 15 000 × *g* for 10 min. The three washed mitochondrial fractions were separately suspended in Isolation Medium to a concentration of 20 mg of protein per ml.

For protein assays, the method of Lowry et al. [5] was used.

Respiration and oxidative phosphorylation

Oxygen consumption by mitochondrial preparations was measured polarographically with a Gilson Model KM Oxygraph calibrated by the procedure of Billiar et al. [6]. Results are expressed as nanogram atoms of oxygen consumed/min per mg mitochondrial protein. Respiratory states are designated by the terminology of Chance and Williams [7]. Respiratory control ratios and ADP/O ratios were calculated from measurements of state 3 and state 4 respiration rates as described by Estabrook [8].

All incubations were carried out at 30 °C in 2 ml of a standard medium consisting of 15 mM Tris · HCl (pH 7.4), 10 mM potassium phosphate (pH 7.4), 1.5 mM EDTA, 0.13 M sucrose, bovine serum albumin (2 mg/ml) and 6 mM $MgCl_2$. The amounts of mitochondria, substrates, ADP or other additions used for individual experiments are given in Results.

Inorganic phosphate metabolism

Uptake of inorganic phosphate or its release into the medium during incubations were determined by similar procedures. Incubations were stopped by the addition of 0.2 ml of 65 % trichloroacetic acid followed by 6.8 ml of 5 % tricholoroacetic acid. Water was added to adjust the final volume to 10 ml and the mixture was centrifuged for 10 min at 6000 rev./min. The supernatant solution was then assayed for inorganic phosphate by the method of Martin and Doty [9]. Unincubated mixtures without substrate and incubated mixtures without mitochondria served as controls.

ATPase assay (inorganic phosphate release from ATP)

Incubation conditions were modified from the procedure of Kielley [10]. Incubation mixtures contained the following: 5 mM ATP, 30 mM KCl, 0.25 M sucrose, 1.5 mM EDTA, 2 mg/ml bovine serum albumin, 40 mM histidine (pH 7.5), mitochondria (1 mg protein/ml). Preincubation of the mixture was carried out for 5 min prior to the addition of mitochondria. Incubation was then continued at 30 °C for 10 min.

"ADPase" assay (release of inorganic phosphate from ADP)

This assay measures phosphate released by true adenosine 5'-diphosphate phosphohydrolase, phosphate release through the sequential action of adenylate kinase (EC 2.7.4.3) and ATPase (EC 3.6.1.3) and phosphate released through the sequential activities of adenylate kinase, ATP pyrophosphohydrolase (EC 3.6.1.8) and inorganic pyrophosphatase (EC 3.6.1.1). All of these activities have been reported to be present in the human term placenta [11].

ADP (4 mM) was incubated at 30 °C for 20 min with 2 mg mitochondrial protein in 1.5 ml of the standard medium used for studies of oxidative phosphorylation.

RESULTS

Preparation and fractionation of mitochondria

Using the procedures described under Materials and Methods, placental mitochondria were prepared and separated into three fractions. Approximately equal amounts (30 mg of mitochondrial protein/100 g of placenta) of each fraction were obtained.

Phosphatase activities of mitochondrial fractions

Schreiner and Villee [2] encountered difficulties in their study of ATP formation during oxidative phosphorylation by placental mitochondria because of ATPase and "ADPase" activities present in their preparations. We also experienced this problem in initial studies using unfractionated mitochondria and, therefore, measured these activities in the three fractions. The results are given in Table I.

ATPases, endogenous, Mg^{2+}-activated and 2,4-dinitrophenol-activated, were lowest in the heavy mitochondrial preparation with increasing amounts of all activities found in the lighter fractions. A similar pattern of distribution was found with "ADPase" activities but with none detected in the heavy fraction.

TABLE I

PHOSPHATASE ACTIVITIES OF MITOCHONDRIAL PREPARATIONS FROM HUMAN TERM PLACENTA

The incubation conditions for ATPase and "ADPase" assays and the procedure for measurement of the inorganic phosphate released are described in Materials and Methods. Dinitrophenol (DNP) was used at a concentration of 0.1 mM; NaF at a concentration of 60 mM, and $MgCl_2$ at a concentration 6 mM. Results with ATP are the average of 2 experiments and those with ADP the average of 5 experiments.

Substrate	Additions	Inorganic phosphate released (nmol/min per mg protein)		
		Fraction: I	II	III
ATP		10	19	31
ATP	Mg^{2+}	87	102	210
ATP	Mg^{2+} + DNP	115	150	262
ADP		0	100	190
ADP	F^-	—	0	30

The observed differences in the phosphatase activities of these preparations could reflect true differences in endogenous mitochondrial activities, differences in activation of latent enzymes by preparative procedures or differences in the degree of contamination with lighter particulate material, such as microsomes. From an experimental standpoint, however, it is apparent that separation of heavy mitochondria from lighter fractions could provide a partial solution to the problem of interference by ATPase and "ADPase" activities in studies of oxidative phosphorylation by placental mitochondria.

Respiration of mitochondrial fractions

Limited oxygen consumption (5–10 ng atoms oxygen/min per mg protein) occurred with all mitochondrial fractions in the absence of exogenous substrates. Addition of ADP failed to stimulate this endogenous respiration. Succinate or ascorbate plus TMPD stimulated respiration in all fractions but NAD-linked substrates gave little or no stimulation of oxygen uptake above endogenous levels, even in the presence of exogenous NAD. When ADP was added in the presence of glutamate, isocitrate, α-ketoglutarate or pyruvate plus malate, however, respiration was stimulated with a transition from state 4 to 3. This permitted the demonstration of the utilization of these substrates and the calculation of respiratory control ratios. Results obtained with the heavy fraction are given in Table II. Rates of respiration by these mitochondria exceeded those previously reported for unfractionated placental mitochondria [1–3]. In addition, respiratory control was demonstrated to be operative at all three sites of energy conservation.

Qualitatively similar patterns of substrate oxidation were also observed with both of the lighter mitochondrial preparations. Lower rates of oxidation and lower respiratory control ratios were generally observed with these fractions. However, the "ADPase" activities of these fractions prevented a valid quantitative assessment

TABLE II

RESPIRATION OF PLACENTAL HEAVY MITOCHONDRIA

Measurements of oxygen consumption and calculation of respiratory control ratios and ADP/O ratios are described under Materials and Methods. Incubation mixtures contained 2 mg heavy mitochondrial protein in 2 ml of standard medium. Substrates were added to a final concentration of 10 mM to achieve state 4. ADP was added to a final concentration of 0.25 mM to induce state 3, except that ADP was 0.05 mM when ascorbate-TMPD was used as substrate. Results are the average of three experiments. ADP/O ratios were measured in separate experiments in the presence of 9 mM sodium fluoride.

	Oxygen consumption (ngatoms/min per mg protein)		Respiratory control ratio	ADP/O
	State 4	State 3		
Pyruvate + malate	9	19	2.1	2.2
α-Ketoglutarate	11	24	2.2	2.8
Isocitrate	10	19	1.9	2.5
Glutamate	7	20	2.9	2.3
Succinate	25	68	2.7	1.8
Ascorbate + TMPD	29	39	1.3	0.9

TABLE III

EFFECT OF NaF ON RESPIRATION AND PHOSPHORYLATION BY MITOCHONDRIAL PREPARATIONS FROM HUMAN TERM PLACENTA

Incubation conditions and calculations are the same as in Table II, S3 and S4 represent states 3 and 4 respectively. RCR is the respiratory control ratio. Results are the average of two experiments.

Substrate	NaF (mM)	Mitochondrial preparations											
		Fraction I				Fraction II				Fraction III			
		Oxygen uptake (ngatoms/min per mg protein)				Oxygen uptake (ngatoms/min per mg protein)				Oxygen uptake (ngatoms/min per mg protein)			
		S4	S3	RCR	ADP/O	S4	S3	RCR	ADP/O	S4	S3	RCR	ADP/O
Glutamate	0	10	40	4.0	—	6	20	3.3	—	8	26	3.5	
Glutamate	20	9	20	2.2	2.9	5	13	2.6	2.1	6	16	2.6	1.7
Glutamate	40	8	13	1.7	3.7	5	9	1.8	2.1	5	12	2.4	2.5
Succinate	0	30	76	2.5	—	18	47	2.6	—	22	55	2.4	—
Succinate	20	16	52	3.2	1.5	14	35	2.5	1.0	15	47	3.2	0.9
Succinate	40	14	35	2.5	1.2	9	22	2.4	1.2	12	28	2.6	1.0
Ascorbate-TMPD	0	28	35	1.3	1.1	21	21			22	20		
Ascorbate-TMPD	20	25	30	1.2	1.2	18	21			20	21		
Ascorbate-TMPD	40	29	26			22	20			22	21		

of the stimulatory effects of ADP on respiration by these mitochondria and for this reason these data are not included in Table II.

No stimulation of respiration by β-hydroxybutyrate occurred with any fraction either in the presence or absence of ADP, and no production of acetoacetate from this compound could be detected by the method of Walker [12].

Conditions for the assay of oxidative phosphorylation

Use of fluoride. In the experiments reported in Table II stimulation of oxygen consumption of heavy mitochondria by ADP indicated a coupled condition but estimation of the efficiency of energy conservation could not be made since state 3 respiration was maintained, until the oxygen supply was exhausted, by a constantly regenerated supply of ADP provided by ATPase activities. In some incubations with fraction III, a re-establishment of state 4 respiration rates was eventually observed but this was due to ADP removal caused by "ADPase" activities and therefore an invalid basis for calculations of ADP/O ratios. The ADP/O ratios listed in Table II were calculated from data from parallel incubations containing 9 mM NaF in which state 4 respiration was re-established. Measurements of inorganic phosphate confirmed that this was accompanied by phosphorylation and not hydrolysis of ADP. However, this level of F^- did not limit the phosphatase activities of lighter mitochondrial fractions sufficiently to permit measurements of coupled phosphorylation by the mitochondria in these fractions. A more detailed study of the effect of F^- on oxidative phosphorylation in placental mitochondrial fractions was, therefore, carried out, the results of which are given in Table III.

In the presence of appropriate levels of F^-, ADP-induced state 3 respiration rates did not persist and respiration did revert to the original state 4 levels, permitting the calculation of ADP/O ratios for all three mitochondrial preparations. Simultaneous measurements of inorganic phosphate uptake gave P/O ratios which correlated well with the ADP/O ratios and confirmed that "ADPase" and ATPase activities had been effectively controlled. With heavy mitochondria, ADP/O values approached the theoretical maxima for the substrates tested, indicating efficient coupling at all three sites of energy conservation. With both lighter fractions, however, ADP/O ratios (and P/O ratios) of approximately one less than the theoretical maxima were obtained indicating a partial uncoupling of these mitochondria. This defect was localized to the terminal coupling site (III) by the finding of ADP/O and P/O ratios of 1 using succinate and of zero with ascorbate-TMPD as substrate.

Assays in the absence of fluoride. While the use of fluoride to control phosphatase activities was successful in permitting the quantitation of ATP production by the polarographic assay it was not without disadvantages. Rates of respiration, particularly in state 3 were depressed by F^- resulting in reduced respiratory control ratios (Table III). Less serious was the complication that optimum concentrations of fluoride varied with the mitochondrial fraction tested. Incubation conditions were, therefore, sought which would permit polarographic measurements of both respiratory control and ATP formation in the absence of fluoride. Appropriate conditions were developed in a study in which both ADP and mitochondrial concentrations were varied. These assay conditions were then utilized to characterize further the three mitochondrial fractions, as reported in Table IV. These data confirm the previous findings of the more limited study in the presence of fluoride. These data also

TABLE IV

RESPIRATION AND PHOSPHORYLATION BY THREE FRACTIONS OF HUMAN TERM PLACENTAL MITOCHONDRIA

Incubation mixtures contained 1 mg mitochondrial protein in a final volume of 2 ml of standard medium. Endogenous respiration was monitored for 6 min and then state 4 respiration was induced with 10 mM glutamate, succinate or ascorbate. With the latter, 0.12 mM TMPD was also included. After 4 min, state 3 respiration was achieved with the addition of 0.05 mM ADP to incubations with glutamate and succinate and with 0.025 mM ADP to incubations with ascorbate-TMPD. Results are the average of 7 or 8 experiments. Variability is expressed in terms of standard error of the mean.

Substrate	Mitochondrial fraction	Oxygen uptake (ngatoms/min per mg protein)		Respiratory control ratio	ADP/O
		State 4	State 3		
Glutamate	I	9.3±0.53	32.4±0.66	3.65±0.22	3.01±0.07
Glutamate	II	7.6±0.52	20.9±1.27	2.83±0.27	1.91±0.07
Glutamate	III	7.6±0.21	22.3±1.15	2.93±0.13	1.81±0.08
Succinate	I	26.6±3.0	61.3±6.16	2.41±0.19	1.98±0.05
Succinate	II	18.3±2.2	34.0±3.08	1.90±0.12	1.11±0.14
Succinate	III	18.4±2.7	33.4±4.05	1.78±0.08	1.05±0.05
Ascorbate-TMPD	I	44.9±2.2	58.5±2.00	1.31±0.04	1.07±0.05
Ascorbate-TMPD	II	41.6±1.8	42.1±1.85		
Ascorbate-TMPD	III	47.0±1.6	46.3±1.72		

indicate that the isolation and assay procedures developed are capable of providing reproducible preparations of mitochondria which display consistent capacities for oxidative phosphorylation.

Evaluation of modified isolation procedures. These isolation and assay procedures were developed prior to the appearance of the report by Klimek et al. [3] on the protective effect of bovine serum albumin on placental mitochondria. We subsequently tested whether use of the techniques of these authors would further improve our preparations. Addition of 1 % albumin to both our Isolation and Resolving Media produced no change in the total yield of mitochondria, in the distribution of mitochondria between the three fractions or in the quantitative parameters of respiration and phosphorylation with glutamate, succinate or ascorbate-TMPD as substrate. Utilization of 0.01 M Tris (pH 7.4) employed by Klimek et al. [3], as buffering agent in both our Isolation and Resolving Media did increase the total yield of mitochondria and resulted in a larger proportion of the mitochondria appearing in the heavy fraction. However, all three fractions displayed poorer indices of oxidative phosphorylation. State 3 respiration rates were reduced resulting in lowered respiratory control ratios and ADP/O ratios were also less than those of our standard preparations. Addition of 1 % albumin to Tris-buffered media did improve somewhat the ADP/O ratios of all three fractions, as observed by Klimek et al. [3] with unfractionated mitochondria, but failed to protect against the effect of Tris on the respiratory control ratios. Albumin in the presence of Tris did selectively increase the yield of light mitochondria, suggesting that these mitochondria might be equivalent to the "type a" mitochondria which predominated in the albumin-processed preparations of Klimek et al. [3]. However, both lighter fractions prepared by any of these procedures were consistently uncoupled at site III.

DISCUSSION

Early studies by Villee and associates [1, 2] of respiration and ATP formation by the human placenta indicated a generally poor performance of these functions by the mitochondria from term placentas. However, the occasional occurrence of more active preparations [1] and the experimental uncertainties caused by the presence of potent phosphatase activities [2] suggested to these workers that mitochondria from this tissue might not necessarily be intrinsically inferior or defective. Klimek et al. [3] recently presented evidence in support of this supposition. By the addition of bovine serum albumin to placental homogenates prior to mitochondrial isolation, they were able to improve the P/O ratios of their preparations to approach the theoretical maximum values, but found, however, no significant improvement in respiration.

In the present study, we have developed an experimental approach to evaluating placental mitochondria combining mitochondrial fractionation and modification of assay conditions to eliminate or minimize interference arising from ATPase or "ADPase" activities.

Heavy placental mitochondria, prepared and tested by the procedures developed, did indeed display properties of active mitochondria. A number of substrates were oxidized at rates significantly greater than those previously reported for unfractionated placental mitochondria. Respiratory control measurements indicated coupling of ATP formation to respiration at three sites, and ADP/O ratios approaching theoretical values demonstrated efficient energy conservation at these coupling sites.

Light mitochondria also carried out respiration and coupled phosphorylation. While respiration rates were generally less than those observed with heavy mitochondria, they still exceeded previously reported values for unfractionated mitochondria. Respiratory control was also less marked in this fraction. Most striking, however, was the apparently selective uncoupling at site III.

The obtaining of a third, less dense fraction of placental mitochondria was unexpected, especially since gentle preparative procedures were employed. From their close resemblance to light mitochondria in oxidative parameters, including the uncoupled state of site III, these particles appeared to be a more extreme form of light mitochondria. The difference in phosphatase activities of these preparations may reflect only differences in microsomal contamination.

Thus the early premise [1, 2] that placental mitochondria are not necessarily deficient in regards to their performance of oxidative phosphorylation has been proven of be correct in the case of the heavy fraction. But the finding of a larger proportion of particles, isolated in fractions II and III, which are partially uncoupled, indicates that the presence of phosphatase activities was not the sole reason for the poor capacity for oxidative phosphorylation displayed by unfractionated placental mitochondrial preparations.

Human placental mitochondria have been previously found to resemble those of other steroid hormone-producing, endocrine tissues in possessing an alternative pathway for the utilization of reducing equivalents and oxygen via the cytochrome P-450-catalyzed conversion of cholesterol to pregnenolone [13–16]. In the current study, the capacity for oxidative phosphorylation of placental mitochondria was also found comparable to that of mitochondria from other steroidogenic tissues. Rates of respiration, particularly with succinate, respiratory control ratios and ADP/

O ratios of heavy placental mitochondria were all similar to those reported for mitochondria from bovine and rat adrenal cortex [17–19] and bovine and porcine corpus luteum [20, 21].

Morphological heterogeneity of human placental mitochondria has previously been observed with the electron microscope to exist in situ [3, 22] as well as in isolated mitochondrial preparations [3, 23]. Klimek et al. [3] described two principal morphological forms in their mitochondrial preparations and Corash and Gross [23] separated placental mitochondria into two fractions in sucrose density gradients. No biochemical differentiation of these fractions was attempted, however.

The preponderance of lighter, partially uncoupled particles in our mitochondrial preparations, despite the use of gentle processing techniques, requires some explanation since light mitochondrial have customarily been considered to be damaged and swollen structures to be removed and discarded. These structures could occur in situ as normal components of a senescent, terminal tissue, or could arise as artifacts from a distinct population of mitochondria, more vulnerable to the anoxic conditions of delivery [3] or to the experimental processes required for their isolation [23].

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REFERENCES

1 Villee, C. A. and Hagerman, D. D. (1961) Gen. Comp. Endocrinol. 1, 317–321
2 Schreiner, W. and Villee, C. (1965) Am. J. Obstet. Gynecol. 91, 961–966
3 Klimek, J., Aleksandrowicz, Z., Wrzolkowa, T. and Zelewski, L. (1972) Biochem. Med. 6, 426–437
4 Green, D. E. and Ziegler, D. M. (1963) in Methods in Enzymology (Colowick, S. P. and Kaplan, N. O., eds), Vol. VI, pp. 416–424, Academic Press, N.Y.
5 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
6 Billiar, R. B., Knappenberger, M. and Little, B. (1970) Anal. Biochem. 36, 101–104
7 Chance, B. and Williams, G. R. (1955) J. Biol. Chem. 217, 409–427
8 Estabrook, R. W. (1967) in Methods in Enzymology (Estabrook, R. W. and Pullman, M. E., eds) Vol. X, pp. 41–47, Academic Press, N.Y.
9 Martin, J. B. and Doty, D. M. (1949) Anal. Chem. 21, 965–967
10 Kielley, W. W. (1955) in Methods in Enzymology (Colowick, S. P. and Kaplan, N. O., eds), Vol. II, pp. 593–595, Academic Press, N.Y.
11 Cerletti, P. and Zichella, L. (1960) Clin. Chim. Acta 5, 748–752
12 Walker, P. G. (1954) Biochem. J. 58, 699–704
13 Morrison, G., Meigs, R. A. and Ryan, K. J. (1965) Steroids Suppl. II, 177–188
14 Ryan, K. J., Meigs, R. and Petro, Z. (1966) Am. J. Obstet. Gynecol. 96, 676–682
15 Meigs, R. A. and Ryan, K. J. (1968) Biochim. Biophys. Acta 165, 476–482
16 Mason, J. I. and Boyd, G. S. (1971) Eur. J. Biochem. 21, 308–321
17 Cammer, W. and Estabrook, R. W. (1967) Arch. Biochem. Biophys. 122, 721–734
18 Billiar, R. B., Alousi, M. A., Knappenberger, M. H. and Little, B. (1971) Arch. Biochem. Biophys. 144, 30–50
19 Sauer, L. A. and Mulrow, P. J. (1969) Arch. Biochem. Biophys. 134, 486–496
20 McIntosh, E. N., Uzgiris, V. I., Alonso, C. and Salhanick, H. A. (1971) Biochemistry 10, 2909–2916
21 Robinson, J. and Stevenson, P. M. (1971) Eur. J. Biochem. 24, 18–30
22 Wynn, R. M. (1972) Am. J. Obstet. Gynecol. 114, 339–355
23 Corash, L. and Gross, E. (1973) Pediat. Res. 7, 798–811