THE NINHYDRIN REACTION OF UNHYDROLYSED PHOSPHOLIPIDS

by

C. H. LEA AND D. N. RHODES

Low Temperature Station for Research in Biochemistry and Biophysics, University of Cambridge, and Department of Scientific and Industrial Research, Cambridge (England)

A sensitive method for the estimation of free amino groups in unhydrolysed phospholipids, even if only approximate, would be of considerable value in connexion with chromatographic and other methods now being developed for the separation and purification of phospholipids from natural sources (*cf.* LEA AND RHODES[1,2,3]). In a previous paper (LEA AND RHODES[4]) the ninhydrin in buffered methyl cellosolve reagent used by MOORE AND STEIN[5] for the determination of amino acids was applied, with suitable modifications to maintain the lipids in solution, to the estimation of amino groups in egg phospholipid.

Since completion of that work improved versions of the ninhydrin procedure for amino acids have been described (TROLL AND CANNAN[6]; COCKING AND YEMM[7]) which increase the rather low and variable molar colour yields obtained in the MOORE AND STEIN procedure to give with most amino acids stoichiometric yields of diketohydrindylidenediketohydrindamine (DYDA), the coloured end-product of the reaction. In addition, supplies of amino-containing phospholipids, both natural and synthetic, of wider variety and higher purity than those previously used had become available and it seemed desirable to attempt to improve the method as applied to phospholipids and, at the same time, to record the results obtained on the new substrates.

Modification of the ninhydrin procedure on the lines found effective for amino acids, however, did not lead to improved results with phospholipids and the method has been changed only in detail. The molar colour yields obtained from the various amino-containing phospholipids examined showed a considerable scatter, due apparently to decomposition of some of the synthetic materials in the interval between preparation and use, but it was found possible to determine the molecular extinction for phosphatidylethanolamine fairly accurately and to obtain approximate values for ethanolamine plasmalogen and phosphatidylserine.

Phospholipids containing free amino groups are notoriously unstable and, until very recently, have been difficult to prepare in anything approaching a pure condition. One of the purposes for which the present procedure has been devised is an investigation of the deteriorative changes which these substances undergo during storage.

EXPERIMENTAL

Ninhydrin methods used

Present paper (LEA AND RHODES[4], modified as indicated). The solvent, usually chloroform or ethanol, is rapidly removed at a low temperature from phospholipid samples containing 1–5 μg

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amino N in 5 × $^5/_8$in. test tubes, conveniently by use of a simple rotary film vacuum evaporator (LEA, HANNAN AND RHODES[8]). The residual lipid is taken up in 1 ml redistilled methyl cellosolve, 1 ml of the ninhydrin-citrate-stannous chloride in 50% methyl cellosolve reagent of MOORE AND STEIN[5] is added and the tubes, fitted with slip-on aluminium caps, are heated in a boiling water bath for 20 min. After cooling, 5 ml methyl cellosolve containing 10% water are added and the optical density of the solution (volume 7.0 ml) is measured in 1 cm cell. Throughout the present work readings have been taken at 575 mμ with a Uvispek spectrophotometer, in place of an instrument selecting a much wider waveband as used in the earlier work[4], slightly higher values and greater sensitivity being thereby obtained. The simpler instrument may, however, be used if desired. The ninhydrin reagent was prepared from recrystallized ninhydrin in 1 l batches, de-aerated and stored under nitrogen with protection from light, as previously described. The methyl cellosolve was redistilled from stannous chloride and stored in the dark. A "blank" determination is carried out without the phospholipid.

Dilution with 90% methyl cellosolve (10% v/v water) has been adopted in the present work in place of pure cellosolve because, as shown below, DYDA under these conditions gives a slightly higher m*M* extinction. If any difficulty should be experienced with exceptionally insoluble lipid failing to remain in clear solution dilution with pure methyl cellosolve, as described in the earlier method[4], may be used.

COCKING AND YEMM[7]. The method was used as described for amino acids and was also tested in a form modified for use with phospholipids by dissolving the sample in 1 ml methyl cellosolve (instead of water) and by diluting the reaction mixture after heating with 5 ml methyl cellosolve (instead of 60% ethanol).

Other analytical methods

Amino N. The free amino N contents of the various fractions isolated from natural sources were determined in acid hydrolysates by the Van Slyke manometric method after removal of the fatty acids by extraction with petroleum ether. The synthetic materials were not available in sufficient quantity to permit the use of this method.

Ethanolamine. Ethanolamine was determined in hydrolysates by the fluorodinitrobenzene (FDNB) technique of AXELROD, REICHENTHAL AND BRODIE[9], which is a micro-technique and could be applied to all samples.

Since some destruction of ethanolamine and serine is liable to occur during alkaline hydrolysis (ARTOM[10]) hydrolysis with 6 *N* HCl for 6–8 h, or in some cases 16 h at 105°, followed by removal of the acid *in vacuo* over NaOH was substituted for the alkaline hydrolysis used by AXELROD *et al.* Aqueous acid appears to be preferable to methanolic HCl for this purpose (LEA AND RHODES[4]), although there seems to be some doubt as to the most suitable duration for the hydrolysis. GERTLER, KREAM AND BATURAY[11] have recently found 24 or 48 h with 6 *N* HCl at 100° optimal for serum phospholipids and have used the latter period, although recovery of the bases under these conditions was slightly low (ethanolamine = 94%; serine = 93–97%). With the somewhat milder conditions described above, any loss of ethanolamine appeared to be within the limits of error of the FDNB determination.

Colour yields by the ninhydrin method on the unhydrolysed phospholipids have been calculated primarily on the basis of the amino N content of the hydrolysates. The ratio of the amino N content of the hydrolysate to P content gives some indication of the purity of the preparations.

Serine. The serine content of an acid hydrolysate of the synthetic phosphatidylserine was determined by the method of MOORE AND STEIN[5] calibrated against the pure amino acid.

Choline. Choline was determined in hydrolysates of some of the egg phosphatidylethanolamine preparations by the periodide method of APPLETON, LA DU, LEVY, STEELE AND BRODIE[12].

Phospholipid preparations used

Egg phospholipid. Crude hen's-egg phospholipid, prepared and purified from contaminating amino acids as previously described (LEA AND RHODES[4]) contained approx. 19% amino N/P.

Egg phosphatidylethanolamine. Several preparations of egg phosphatidylethanolamine were obtained from hen's-egg phospholipids by chromatographic separation on a silicic acid column (LEA AND RHODES[1,3]). The products showed only one spot on silicic acid paper (LEA AND RHODES[2,3]) and, after hydrolysis and removal of the fatty acids gave an amino N/P ratio of 0.98–1.00. All gave slight positive reactions for choline by the periodide method[12]. The choline values obtained for egg preparation No. 3 (1.1 and 1.5%) were not appreciably higher than the apparent choline contents by this method of two of the synthetic substances (0.4 and 1.3%) and are probably not significant. The slightly higher values (not determined accurately) of the earlier egg phosphatidylethanolamine samples may have indicated the presence of a little choline as impurity but the amounts can only have been very small in view of the amino N figures.

Synthetic phosphatidylethanolamines. The dimyristoyl-L-α-compound was kindly supplied by Professor E. BAER, the dipalmitoyl-DL-α- by Dr. T. MALKIN and the dipalmitoyl-β- and palmitoyl-linoleoyl-DL-β- compounds by Dr. W. G. ROSE.

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Synthetic ethanolamine plasmalogen (2-aminoethyl 2:3-o-hexadecylidene-1-glycerophosphate). A sample of this substance was kindly supplied by Dr. T. MALKIN.

Brain phosphatidylserine. Two preparations of phosphatidylserine obtained from ox brain by the method of FOLCH[13] gave amino N/P ratios of 0.97 and 1.00 and ethanolamine N/total amino N ratios of 0.02 and 0.04. Treatment of the hydrolysates with an ion-exchange resin showed that practically all of the amino N was acidic. The material, however, after catalytic hydrolysis with $HgCl_2$ gave a strong reaction for aldehyde with Schiff's reagent and was apparently still contaminated with plasmalogen, as also found by LOVERN[14].

Synthetic phosphatidylserine. A sample of the distearoyl-L-α-phosphatidyl-L-serine was kindly supplied by Professor E. BAER.

None of the synthetic substances, with the possible exception of the plasmalogen, was chromatographically homogeneous when run in methanol-chloroform on silica-impregnated paper (LEA AND RHODES[2,3]), although the saturated β-compound was very nearly so. All except the plasmalogen, however, had been prepared a considerable time before use and Dr. BAER advised us that the phosphatidylethanolamine supplied by him had suffered "about 10%" decomposition. The unsaturated phosphatidylethanolamine was available only in very small quantity and had obviously undergone some autoxidative decomposition.

RESULTS

Influence of solvent on colour yield

Preliminary tests with glutamic acid showed that the m*M* extinction of 21.4 (= 99% DYDA) given by this substance by the COCKING AND YEMM[7] method became appreciably smaller as the water present in the reaction mixture and in the solvent used for dilution was replaced by methyl cellosolve. It was therefore decided to base all results on the colour given by pure DYDA in the particular solvent system being used. The absorptions of pure DYDA sodium salt, prepared from glycine as described by MOORE AND STEIN[5] and dried *in vacuo*, are given in Table I. To obtain these data the reagents after heating *etc.* as in the determination were added to tubes containing the dry DYDA.

TABLE I

COLOURS GIVEN BY PURE DYDA UNDER VARIOUS CONDITIONS

Method	*Final volume (ml)*	*% water (v/v) in reaction mixture*	*% water (v/v) in final solution*	*mM extinction at 575 mμ*
COCKING AND YEMM[7]	7.7	56	45	21.6
Modified COCKING AND YEMM	7.7	19	7	19.7
Present paper (100% cellosolve)	7.0	25	7	20.8
Present paper (90% cellosolve)	7.0	25	14	21.6

The method described by LEA AND RHODES[4], but using a spectrophotometer, (referred to in the tables as "present paper, 100% cellosolve") gave a m*M* absorption for DYDA of 20.8 which could be increased to a maximum value of 21.6 by diluting the mixture after reaction with 5 ml of 9:1 (v/v) methyl cellosolve-water in place of pure methyl cellosolve (Table I). As shown below, the m*M* extinction given by egg phospholipid bore the same relation to that of DYDA measured in the same solvent whichever variant of the method was used.

Comparison of procedures

Table II gives a comparison of the methods using serine, ethanolamine hydrochloride and a sample of egg phospholipid. Increasing the times of heating beyond those specified in the methods did not raise the values obtained. Since the method of COCKING

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AND YEMM modified for use with phospholipids gave lower values than that of LEA AND RHODES the latter was retained for use with phospholipids and subsequent determinations were all carried out with this method, modified as already indicated by use of the spectrophotometer for measurement of the colour produced and (optionally) of aqueous cellosolve for dilution.

TABLE II

MOLAR COLOUR YIELDS OBTAINED BY THE SEVERAL METHODS

Substance	*Method*	*mM extinction at 575 mμ*	*as % DYDA in the same solvent*
Serine	COCKING AND YEMM[7]	19.8	92
	Modified COCKING AND YEMM	15.2	77
	Present paper (100% cellosolve)	18.2	87
	Present paper (90% cellosolve)	18.4	85
Ethanolamine	COCKING AND YEMM	3.8	18
	Modified COCKING AND YEMM	9.4	48
	Present paper (100% cellosolve)	15.0	72
	Present paper (90% cellosolve)	13.8	64
Egg phospholipid	Modified COCKING AND YEMM	16.8	85
	Present paper (100% cellosolve)	18.6	89
	Present paper (90% cellosolve)	19.3	89

* Calculated on total N = amino N basis for serine and ethanolamine, and on the Van Slyke amino N content of a hydrolysate for egg phospholipid.

The absorption curve for pure DYDA or for the colour produced by egg phospholipid in the final solvent mixture is almost flat between 570 and 580 mμ, and reading at 575 mμ has been standardized. Beer's law is obeyed over a reasonable range of concentrations and plots of optical density against concentration have not, therefore, been given.

Although results on serine and ethanolamine by the method of the present paper are included in Table II for purpose of comparison, reproducibility with these water-soluble compounds, particularly ethanolamine, is poorer than with phospholipids, and the method is not recommended for use with these substances. With egg phospholipid the average of 8 determinations diluting with 100% methyl cellosolve was 89.5% DYDA ± 1.0 (S.D.), and of 11 determinations diluting with 90% methyl cellosolve 89.0 ± 0.6%. The latter series of determinations was carried out on the same stock lipid solution stored at −30° C over a period of 6 weeks, with two batches of ninhydrin reagent (made from the same stock of recrystallised ninhydrin). At this order of reproducibility the determination of amino N in the intact phospholipid is not inferior to the corresponding determination by micro methods in a hydrolysate.

Colour yield of amino-containing phospholipids

Phosphatidylethanolamines. The results which have been collected in Table III show that a sample of egg phospholipid containing approximately 19% phosphatidylethanolamine gave a molar colour yield for this substance in the direct ninhydrin determination of 86 or 89% DYDA. The discrepancy in the figures depends on the slightly different values obtained for the amino N and ethanolamine N contents of the hydrolysate by the Van Slyke and FDNB (micro) methods respectively, a difference which was not

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resolved by further determinations. If the figure obtained by the Van Slyke procedure is preferred then 89% is the correct value.

TABLE III

NINHYDRIN REACTION OF UNHYDROLYSED PHOSPHOLIPIDS*

Substance	Amino N in hydrolysate/P		Colour yield of phospholipid (as % DYDA)	
	Van Slyke method (NH_3)	FDNB method (ethanolamine)	Based on P content	Based on amino N content of hydrolysate
Egg phospholipid	0.186	—	—	89
Egg phospholipid	—	0.194	—	86
Egg PE, prepn. 1	0.99	0.97	84	86
Egg PE, prepn. 2	0.99	—	85	86
Egg PE, prepn. 3	0.99	1.00	89	89
Egg PE, prepn. 4	1.00	0.99	91	91
Ethanolamine plasmalogen	—	0.98	85	87
Brain PS, prepn. 1	1.00	0.04	91	91
Brain PS, prepn. 2	0.97	0.02	90	93
Distearoyl-L-α-PS	—	0.93**	82	89

* PE = phosphatidylethanolamine; PS = phosphatidylserine.
** Serine by method of MOORE AND STEIN[5].

The isolated egg phosphatidylethanolamine fractions also gave colour yields of 86 to 91% DYDA per amino group, the highest values being obtained from the purest preparations.

The synthetic phosphatidylethanolamines gave disappointingly inconsistent results, the mM extinctions calculated on the P basis ranging from 88% DYDA for the dipalmitoyl-β-compound down to 75% for the dipalmitoyl-DL-α-compound. Determination of ethanolamine in hydrolysates of these substances by the FDNB method, however, showed that all contained less than the theoretical 1.00 molecule of ethanolamine per atom of P, the ratio ranging (in the same order as the direct ninhydrin colour) from 0.97 down to 0.78. Plotting the colour yields in the direct ninhydrin determination against the ethanolamine/P ratio in the hydrolysate, with extrapolation back to a ratio of 1.00 (*i.e.* no decomposition), indicated a probable colour yield for pure phosphatidylethanolamine of approximately 89% DYDA (Fig. 1); linear extrapolation would indicate a somewhat higher figure.

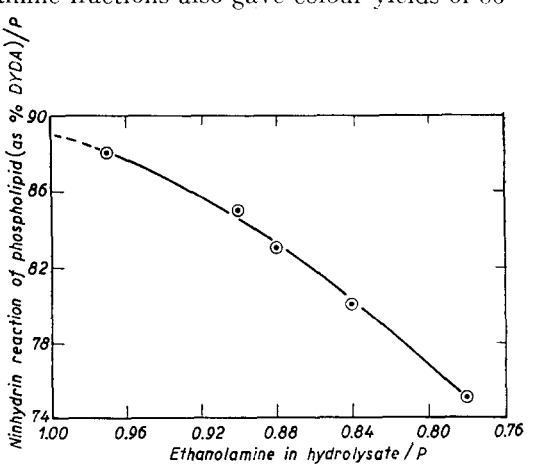

Fig. 1. The relation between the colour developed by a series of synthetic phosphatidylethanolamines in the direct ninhydrin reaction and the ethanolamine content of the hydrolysates as determined by the FDNB method. Reading from left to right: 1 = Dipalmitoyl-β-; 2 = 4 after passage through a silicic acid column; 3 = Palmitoyllinoleoyl-DL-β-; 4 = Dimyristoyl-L-α-; 5 = Dipalmitoyl-DL-α-.

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Plasmalogen. The single sample of ethanolamine plasmalogen available, at 87% DYDA on the ethanolamine N basis was little, if any, different from the phosphatidylethanolamines (Table III).

Phosphatidylserines. The two preparations of phosphatidylserine from brain gave colour yields of 91 and 93% DYDA on the amino N basis, a result only slightly different from that on the synthetic product (89%) which was about 6 months old and had undergone some slight decomposition as indicated both by its low colour yield on the P basis and by its amino N/P ratio of 0.93 (Table III).

DISCUSSION

The mechanism of the reaction with ninhydrin of non-carboxylic amino compounds has not been investigated so thoroughly as that with amino acids, but the nature of the coloured end-product appears to be the same (MACFADYEN AND FOWLER[16]). MOORE AND STEIN[5] gave values of 88% DYDA (94% of the leucine standard used by these authors) for serine and 91% for ammonia, but no figure for ethanolamine. LEVINE AND CHARGAFF[15], whose results are expressed only in arbitrary units, found the molar colour yield of ethanolamine by the MOORE AND STEIN method to range from 49 to 71% of that of serine with different batches of ninhydrin reagent. The KCN methods of TROLL AND CANNAN[6] and COCKING AND YEMM[7] both give very low values for ammonia (33% DYDA) and the latter method an even lower value (18%) for ethanolamine (Table II). According to MOORE AND STEIN the colour yield from ammonia with their method varies somewhat with the concentration of hydrindantin in the reaction mixture, which may vary from batch to batch of reagent. For accurate determination of ammonia they therefore recommend that a control determination on a standard ammonium chloride solution be run simultaneously with the unknown samples. We have found no variation in the colour yield from unhydrolysed phospholipids with several batches of reagent prepared from the same stock of recrystallized ninhydrin, but it is possible that this factor contributes to the rather erratic behaviour of ethanolamine itself in the determination.

Reproducibility of the direct ninhydrin determination on unhydrolysed phospholipids has been found to be quite good and most of the difficulty encountered in the present work has been experienced in characterization of the "pure" phospholipids on which calibration of the ninhydrin determination has to be based. With one exception phospholipids containing only saturated fatty acids (or aldehydes) have been synthesised, the synthesis is laborious and the products deteriorate on keeping. Nevertheless it has been possible to deduce a molar colour yield (*ca.* 90% DYDA) for pure, synthetic phosphatidylethanolamines which agrees well with that obtained from fractions isolated by the silicic acid column technique from the phospholipids of egg yolk. There was no indication in these results of any effect on the colour yield of the nature of the constituent fatty acids present *e.g.* saturated or unsaturated. The figure for the single sample of synthetic ethanolamine plasmalogen available was found to be very similar (87% DYDA) and those for the one synthetic and two natural (brain) phosphatidylserines only slightly higher (89, 91, 93% DYDA). Pending examination of further pure substances it would seem that the error involved in calculating mixed amino-containing phospholipids on the basis of a colour yield of 90% DYDA per free amino group would not be very great.

The nature of the decomposition undergone by amino-containing phospholipids on storage is still not known with any certainty and no attempt has been made at this stage

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to elucidate the mechanism of the reaction. It is of interest that passage of a deteriorated phosphatidylethanolamine (Fig. 1, No. 4) through a short column of silicic acid brought back the product approximately half way along the curve (Fig. 1, No. 2) towards the pure material, but the quantities of the synthetic substances available were too small to permit any serious attempt at purification by this or other means.

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SUMMARY

1. The molar colour yields with ninhydrin in buffered methyl cellosolve solution of a series of amino-containing phospholipids have been determined.

2. Examination of four natural (egg) and five synthetic phosphatidylethanolamine preparations indicated that the best value for the absorption at 575 mμ of this substance is approximately 90% of that of diketohydrindylidene-diketohydrindamine (DYDA). In the two solvents described pure DYDA possesses mM extinctions of 20.8 and 21.6.

3. A single synthetic ethanolamine plasmalogen sample gave a colour yield of 87% and one synthetic and two natural (brain) phosphatidylserines values of 89, 91 and 93% DYDA respectively.

4. On the basis of the results obtained it would appear that a common factor of 90% DYDA would not be greatly in error for the estimation of mixed amino-containing phospholipids in lipid extracts.

RÉSUMÉ

1. On a mesuré les couleurs produites dans une série de phospholipides contenant de groupes NH_2 par l'action de la ninhydrine en solution méthyle cellosolve.

2. L'examen des préparations de phosphatidylethanolamine, dont quatre naturelles (oeuf) et cinq synthétiques, a montré que la meilleure valeur pour l'absorption de cette substance, à 575 mμ, était du 90% environ de celle de la diketohydrindylidene-diketohydrindamine (DYDA). L'ε pour la DYDA pure, avec les deux solvants décrits, était = 20.8 et $21.6 \cdot 10^3$.

3. On a obtenu, d'un seul échantillon de plasmalogène éthanolaminé synthétique, un rendement de couleur du 87%. Les valeurs correspondantes pour les phosphatidylsérines, dont l'une synthétique et les deux autres naturelles (cervelle), étaient respectivement du 89, 91 et 93% de la DYDA.

4. En vue de ces résultats on pense pouvoir considérer un facteur common du 90% de la DYDA comme plus ou moins correct dans l'évaluation des phospholipides mixtes contenant de groupes NH_2.

ZUSAMMENFASSUNG

1. Die durch die Wirkung von Ninhydrin in Methyl-Cellosolve-Lösung bei einer Reihe von NH_2-Gruppen enthaltenden Phospholipiden erzeugten Farben wurden gemessen.

2. Prüfung von vier natürlichen (Ei) und fünf synthetischen Phosphatidyläthanolaminpräparaten ergab als besten Wert für die Absorption dieses Stoffes bei 575 mμ etwa 90% der Absorption von Diketohydrindyliden-diketohydrindamin (DYDA). Für reines DYDA war bei den zwei beschriebenen Lösungsmitteln ε = 20.8 und $21.6 \cdot 10^3$.

3. Mit bloss einer Probe synthetischen Äthanolamin-Plasmalogens wurde ein Farbenertrag von 87% erzielt; eine synthetische und zwei natürliche (Gehirn) Phosphatidylserin-Proben ergaben DYDA-Werte von 89 und 91 bez. 93%.

4. Auf Grund dieser Ergebnisse wird bei der Schätzung gemischter NH_2-Gruppen enthaltender Phospholipide ein gemeinsamer Faktor von 90% DYDA als ungefähr richtig anzunehmen sein.

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