612.744.16:546.185
616-006:546.185

PHOSPHATE IN TUMOURS AND MUSCLE.

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THE fermentation of sugar is a fruitful source of anoxidative energy in muscle, yeast and tumours. Phosphate is concerned in muscle and yeast glycolysis as follows:

(1) Fermentable compounds of hexoses with one and with two molecules of phosphate have been isolated from both.

(2) Inorganic phosphate and adenylpyrophosphate form part of the coferment system necessary for the glycolysis of glycogen by muscle extract, the latter compound also serving in the coferment of yeast [Lohmann, 1931].

(3) Creatine-phosphate is possibly related to this system since the anaerobic breakdown of the pyrophosphate fraction in muscle does not take place until the creatine-phosphate has been exhausted [Eggleton, G. P. and P., 1929].

The question naturally arose whether glycolysis of tumours involved phosphate as in muscle and yeast. Barr, Ronzoni and Glaser [1928] found only a small liberation of free phosphate during glycolysis by surviving tumour, and the addition of phosphate affected neither lactic acid production nor sugar disappearance. Lange and Henning [1928] found that the free phosphate washed out of tumours, surviving in Ringer fluid containing glucose, did not parallel the production of lactic acid.

There is certain indirect evidence in favour of phosphate intermediaries in surviving tumour glycolysis. Lactic acid formation by tumour is inhibited by fluoride [Ewig, 1929; Dickens and Simer, 1929], by amylase [Harrison and Mellanby, 1930], and by monoiodoacetic acid [Harrison and Mellanby, 1931]; in muscle these substances inhibit lactic acid formation by either preventing the synthesis or breakdown of hexosephosphate esters. Neuberg, etc. [1930] found that the residue from Jensen's rat sarcoma, after acetone extraction, formed some free phosphate and methyl glyoxal from magnesium hexosediphosphate. Since Warburg and others [1924] have shown that intact

tumour cells can rapidly form lactic acid from methyl glyoxal, these authors inferred that the lactic acid formation in tumour follows a similar path to that of other glycolytic systems. On the other hand, Harrison and Mellanby [1930] found that surviving mouse carcinoma could not produce lactic acid from added hexosemonophosphate or hexosediphosphates. However, Downes [1929] reported some glycolysis of a "hexosephosphate" by rat sarcoma.

This paper reports further investigation into the possible rôle of phosphate in tumour glycolysis as compared with muscle.

Method.

Crocker Institute 180 mouse tumour was used throughout. Changes on glycolysis in the surviving tissue were followed by comparing the extracts of alternate slices before and after 1 hour's incubation at 37·5° C. From three to six mice supplied between 1 and 2 g. for each of the control and incubated tissue. Slicing of each tumour was complete within about 8 minutes after the death of the mouse. The control slices were immediately placed in ice-cold 5 p.c. trichloroacetic acid solution. The slices for incubation were put in 50 c.c. of isotonic Ringer solution containing 0·015 *M* sodium bicarbonate. The fluid was kept in a Petri dish and continually agitated during incubation. The gas phase was a 5 p.c. solution of CO_2 in air. Aseptic precautions were taken throughout and after incubation were checked by cultures. The tissues, after grinding, were left in the ice chest to extract overnight in 5 p.c. trichloroacetic acid, then centrifuged.

After treating an aliquot part with lime and $CuSO_4$, the lactic acid was measured directly by the Friedemann and Kendall [1929] method using $MnSO_4$ and $KMnO_4$. Phosphorus was measured by Martland and Robison's modification [1926] of the Briggs method.

Considerable difficulty was encountered in preparing a clear trichloroacetic acid extract of tumour tissue. The method finally adopted was to neutralize the acid extract with Na_2CO_3, finally adjusting to *p*H 7·0 by drawing off CO_2. A further slight precipitate was centrifuged down, but even this process did not yield perfectly clear extracts.

Experimental.

Labile phosphate in tumour extracts.

To avoid confusion in the free phosphate estimation of the tumour extracts it was first necessary to investigate the incidence of labile

phosphate. It soon became apparent that there was little, if any, phosphate present that was rapidly hydrolysed by acid. The time curve for the development of colour of the phosphate reading, as compared with standard, was practically a straight line only slightly rising within 40 minutes. This was with extracts from tumour tissue removed from the body within 3 minutes of death and immediately ground in ice-cold trichloroacetic acid solution.

To investigate this slight rise further, the more recent method of Eggleton and Eggleton [1929] for creatine-phosphate was used. The results of four experiments on the tumour are given in Table A. Two

TABLE A. Creatine-phosphate in C 180 mouse tumour.

Experiment	Tissue	Weight g.	"Creatine" P Observed mg.	"Creatine" P mg. per 100 g.	Ba-"soluble ester" mg. per 100 g.	Free P mg. per 100 g.	"Pyro" P mg. per 100 g.	Ba-"insoluble ester" mg. per 100 g.
350	C 180 mouse tumour	1·40	0·0037	1·5	13·3	32	5·4	11·1
351	"	1·23	0·0036	2·0	15·5	15·5	7·6	7·9
360	"	0·87	0·0041	2·3	5·5	29	2·5	21·2
361	"	0·67	0·0039	2·7	3·8	30	3·5	18·1
362	Mouse leg muscle	1·00	0·0467	22·5	20·5	58	34	14
363	"	1·55	0·0615	19·5	18	72·5	39	18

experiments done on mouse leg muscle are given as controls. It will be seen that there is only a small quantity, if any, of a substance with the solubility and lability similar to creatine-phosphate present in C 180 tumour. The observed readings are very near to a blank. There is a definite though variable amount of barium "soluble ester," and barium "insoluble ester" apart from that hydrolysed as pyrophosphate. The "pyrophosphate" recorded is simply the increase in reacting phosphate of the barium insoluble fraction after 7 minutes' boiling in *N* HCl. As Boyland [1930] points out, this would include about one-third of the phosphate of any Harden and Young hexosediphosphate present. Both the pyro- and creatine-phosphate are present in about the same concentration as Eggleton and Eggleton found for testis and uterus.

It is therefore not surprising that little free phosphate increase has been found on incubation of the mouse tumour. However, there are present fractions which correspond to the chief hexose esters so far described from muscle and yeast. The barium soluble "ester" would contain any hexosemonophosphate present, whereas most of any hexosediphosphate would be included in the insoluble residue. Although the tumour extracts contained only negligible quantities of labile phosphorus, it was

possible that intermediary phosphate shifts were catalysed by the acid in the extract. All extracts were therefore kept cold until neutralized.

Phosphate balance in incubated mouse tumour.

Incubation experiments were done on the C 180 mouse tumour with and without adding 0·2 p.c. glucose to the Ringer fluid. By comparing the two it was hoped that any excess synthesis or breakdown of a phosphate intermediary might be demonstrated.

Since the incubation tissue in the Ringer fluid was difficult to weigh, we assumed its weight bore the same ratio to its total phosphate content as the control. The control tissue was weighed and the weight of the incubated tissue computed from its relative phosphate content. On checking this method by weighing and estimating the total phosphate of both the incubated and control, we found the computed and observed results from the alternate slices did not vary by more than 5 p.c. In fact the phosphate per unit wet weight of different tumours did not vary by more than twice this amount.

The neutralized trichloroacetic acid extracts were both made up to 72 c.c. with distilled water and divided into 14 equal parts of 5 c.c. each. On one, the free phosphate was determined, on another the lactic acid, and two more served for duplicate total phosphate estimations after digestion. The total phosphate was also done on the sodium carbonate and trichloroacetic acid precipitates. From the sum of these three total phosphates, the total phosphorus of the tissues was obtained.

In Table B the changes on 1 hour's incubation expressed as mg. per 100 g. wet weight are given. In incubation with added sugar the average free phosphate of six experiments increases by about 5 mg. per 100 g., an amount in excess of any labile phosphate present. For seven lots of tissue incubated without sugar the average free phosphate liberated is over twice this amount. Thus the free phosphate liberated during incubation is greater in the absence of added sugar than when sugar is present. Interpretation of these results is complicated by the fact that as shown in column 2, during incubation of the tumour in Ringer solution, there is also an increase in the total acid soluble phosphate. Although variable in different experiments, this increase averages about the same whether sugar is present or absent in the fluid. This would suggest the increase results from the breakdown of the cells damaged in slicing. The net result of these changes is that there is an increase in the "organic" phosphate during incubation in the presence of sugar which does not occur when sugar is absent.

TABLE B. Changes on incubation of C 130 tumour with and without added glucose (mg. per 100 g. wet weight).

Mouse tumour experiment	Glucose in fluid	(1) Free P increase	(2) Total acid-soluble P increase	(3) "Organic" P increase (2)–(1)	(4) "Organic" P increase from precipitation curve 20 to 70 p.c.	(5) Difference between (3) and (4)	(6) Lactic acid formation	Culture of incubation fluid
252	+	1·7	4·1	2·4	—	—	460	?
304	+	8	16·2	8·2	7·5	0·7	430	Slight growth
377	+	4	15·6	11·6	11·4	0·2	—	No growth
385	+	7	11·4	4·4	3·3	1·1	640	Slight growth
389	+	3	10·1	7·1	7·8	0·7	720	,,
403	+	2·5	12·8	10·3	8·4	1·9	700	No growth
	Av.	4·4	11·7	7·3				
321	0	9·5	6·4	−3·1	—	—	30	No growth
341	0	4·5	4·9	0·4	0·6	0·2	25	?
359	0	9·0	5·6	−3·4	−1·5	1·9	—	Several colonies
367	0	9·5	7·4	−2·1	−1·3	0·8	40	Slight growth
372	0	14·5	11·5	−3·0	−1·1	1·9	30	,,
382	0	22·2	19·6	−2·6	−4·0	1·4	70	No growth
392	0	16·9	16·9	0	—	—	40	Slight growth
	Av.	12·3	10·3	−2				

Average, Free P 21·7, Total acid-soluble P 53·4, Total P 238.

The above results represent merely the balance of all the phosphate changes that occur during incubation. If glycolysis of the tumour does involve phosphate intermediaries, in the absence of added sugar the 2 or 3 mg. of free P produced in excess of the new acid soluble P could not account for the appearance of some 30 mg. of lactic acid from the breakdown of any known hexosephosphate. Either the tumour was able to utilize sugar remaining in the cell or there had been a shift from one type of ester to another, as for example, from a mono- to a di-phosphate form with the liberation of a hexose molecule for glycolysis, but no free phosphate. Further experiments were made to test this point.

Fractional precipitation of barium salts by alcohol.

In view of the variation in properties and function of the various phosphates already isolated from other glycolysing tissues, it was essential that any method adopted for following similar substances in tumour should be as general as possible. The method used depends upon the fact that the barium salts of most of the phosphate compounds isolated from muscle and yeast have fairly characteristic solubilities in different concentrations of alcohol. Thus Ba-hexosediphosphate is insoluble in 10 p.c. alcohol at about *p*H 8, whereas Ba-hexosemonophosphate is soluble in

that concentration but is precipitated by 70 p.c. alcohol from a solution of the same acidity. Likewise Ba-pyrophosphate is insoluble in water whereas the barium salts of creatine-phosphate and adenylic acid are soluble at *p*H 8, that of the latter being precipitated by four volumes of alcohol.

Since it was not known that the phosphates in tumour were similar to any of the above, we adopted the more general plan of estimating the "organic" P precipitated by ten different concentrations of alcohol from 0 to 85 p.c., in the presence of barium. The results were plotted as a curve with the alcohol concentration as the abscissa. By comparing the levels and shape of these curves before and after incubation, the synthesis, or shift from one type of phosphate to another with different solubility, could be detected. In an attempt to determine the curve at a *p*H which would differentiate as many substances as possible, the extracts were brought to *p*H 7.

Fractional precipitation of barium compounds by alcohol was therefore done on the remaining ten aliquot parts of the trichloroacetic acid extracts from the experiments quoted in Table B. To each 5 c.c., four drops of saturated $BaCl_2$ solution were added (about 0·1 g.). A different amount of ethyl alcohol was then added to each to make ten different concentrations from 0 to 85 p.c. by volume. These were left to precipitate overnight in the ice chest, then centrifuged. The clear supernatant fluids were decanted into another set of tubes, evaporated to dryness, and the total P estimated after digestion. By subtracting this value from the total acid soluble P of the duplicate 5 c.c. quantities, the total P of the precipitate from each alcohol fraction was obtained. To obtain the free P, the Ba precipitates were taken up in 5 c.c. of 0·1 *N* acetic acid, 1 c.c. of 10 p.c. sodium sulphate added and the barium sulphate centrifuged off. The free phosphate was then estimated in the supernatant fluids. This procedure yielded a 96 p.c. recovery from an orthophosphate solution of 0·04 mg. P content. The "organic" phosphate precipitated by each alcohol fraction was then found by the difference between the free and the total phosphate. This figure would of course contain such pyrophosphate as was present. Four experiments were done in which the total P was estimated on both the precipitate and the supernatant fluid of each alcohol concentration. Unless grossly contaminated, their sum agreed within the same range as that of the duplicate situations of total acid-soluble P, *i.e.* about 0·002 mg. P.

Typical fractional precipitation curves of experiments on mouse tumour are given in Fig. 1. These curves are for the "organic" phosphate precipitated from the extract before and after incubation and the

results are expressed in mg. P per 100 g. wet weight of tissue. In the case of the tumour incubated with sugar it is seen that the increase in the acid-soluble "organic" phosphate is due to one fraction which is precipitated completely by all alcohol concentrations from about 20 p.c.

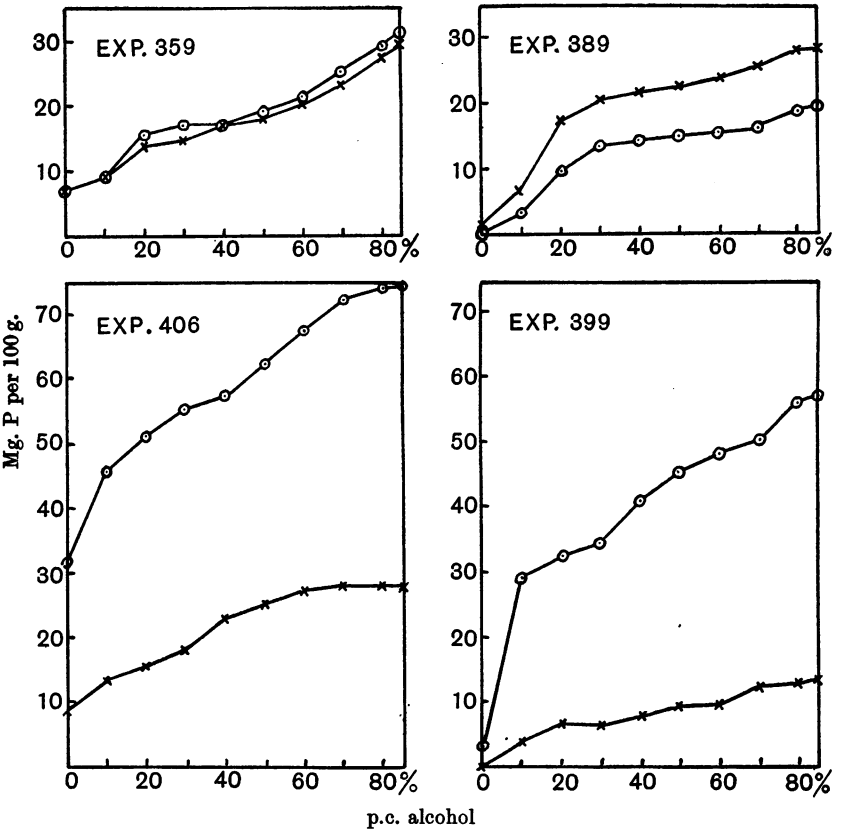

Fig. 1. Fractional barium alcohol precipitation curves for acid-soluble "organic" P expressed in mg. per 100 g. wet weight. Two upper curves are for sliced C 180 mouse tumour. Two lower for minced mouse leg muscle. In two left curves no glucose added to Ringer fluid (0·015 *M* $NaHCO_3$). Two right curves fluid contained 0·2 p.c. glucose. Control (before incubation), 0–0, after incubation (1 hour) *x*–*x*.

up. Apart from this increase the curves are practically parallel. The curves for the tumour incubated without added sugar are parallel and practically coincident. There is no evidence of any increase in the organic P precipitated at 20 p.c.

Since the curves are parallel from 20 p.c. up in tissue incubated with

and without sugar there cannot have been any uncompensated shift from one organic phosphate formed to another in this range: that is, there cannot have been any shift from a hexosemonophosphate to a hexosediphosphate during the incubation for 1 hour, of the C 180 tumour with or without the addition of sugar to the fluid. The accuracy of this statement can be seen from Table B in column 4 of which is tabulated the average change in the organic P precipitated by 20 to 70 p.c. alcohol inclusive. It is seen that this figure varies by less than 2 mg. per 100 g. from the difference between the increase in the acid-soluble P and the increase in the free P as given in column 3. In other words, all the phosphate changes can be accounted for within less than 2 mg. per 100 g. by a free P increase and an "organic" P increase at 20 p.c. alcohol concentration.

Since this organic fraction is all precipitated by 20 p.c. alcohol it behaves as a single substance. Moreover, the increase in free P in the absence of added sugar is either from the breakdown or non-synthesis of a substance having the same property, since not only does the 20 p.c. rise not appear in the curves but there is no other significant change in them to account for the free P balance as given in Table B. If this "organic" substance is a part of the new acid-soluble P derived from the incomplete breakdown of the acid precipitate, then, in the absence of sugar when the energy from glycolysis is much reduced, it is completely broken down to liberate its phosphate; or if it is the result of synthesis, then this synthesis does not take place in the absence of added sugar. It will be noted that this substance has a solubility in keeping with a hexosediphosphate.

Since the increase in the acid-soluble P was at the expense of substances precipitable by trichloroacetic acid, it was of some interest to follow the ether-soluble P. The average ether-soluble P of ten lots of C 180 tumour was 4·7 mg. per 100 g. As the result of 1 hour's incubation this was increased by 3·4 mg. This increase was not significantly affected by the addition of sugar. Mouse muscle under similar conditions showed no change. There is therefore a decrease in the acid-precipitable non-lipoid P of tumour during incubation.

Phosphate in incubated minced muscle.

As a check on the method used in obtaining the above precipitation curves, and by way of comparison with tumour, similar experiments with mouse muscle were made. The animals were killed by stunning as before, and for the control, the muscle of one hind limb quickly removed

and placed in a tared weighing bottle containing 10 c.c. of 5 p.c. trichloro-acetic acid solution in an ice mixture. The muscle of the other hind limb, after mincing finely with scissors, was used for the incubated tissue. Aseptic precautions were taken throughout and cultures were invariably sterile. Apart from the use of acid washed sand in grinding, the technique was as previously described for tumour.

In estimating the free P of the precipitates, readings were made about 40 minutes after adding the sulphuric acid and other ingredients. The results therefore included any labile P present in the precipitates. The soluble Ba-creatine-phosphate was absent from the lower alcohol precipitates. This had to be allowed for. The "organic" values thus obtained correspond to those of the mouse tumour where the labile P was practically negligible, and the free P curves similar to those of Ba-orthophosphate of incubated muscle, *i.e.* maximal at about 30 p.c. alcohol.

The total phosphates and lactic acid for the incubation experiments done on minced mouse muscle are given in Table C. It is seen that the

TABLE C. Incubated minced mouse leg muscle in bicarbonate buffered Ringer's solution with and without glucose.

Experiment	1 hour incubation at 37·5° C.	Tissue weight	Glucose in fluid (0·2 p.c.)	Free P plus labile P		Total acid-soluble P		Lactic acid		Total P of tissue
				Mg. per 100 g.	Increase	Mg. per 100 g.	Change	Mg. per 100 g.	Increase	mg. per 100 g.
399	Control	1·47		88		150		160		
	Incubated	1·68	+	131	43	149	−1	430	270	228
417	Control	0·81		71		129		220		
	Incubated	0·78	+	115	44	134	+5	510	290	237
406	Control	1·50		92		170		160		
	Incubated	1·34	0	153	61	172	+2	270	110	269
409	Control	1·70		80		155		130		
	Incubated	1·46	0	133	53	158	+3	290	160	245

lactic acid produced by the muscle pulp in the absence of added sugar is much greater than with the tumour. The large free P increase (apart from the creatine-phosphate) without any significant increase in the total acid-soluble P is noted in contrast to tumour. This decrease in the "organic" P is reflected in the alcohol precipitation curves. Typical barium fractional curves for the minced mouse muscle before and after incubation are shown in Fig. 1. The results as before are expressed as mg. P per 100 g. wet weight. It is seen that the upper control curves show a definite break at about 30 p.c. alcohol, indicating a fraction completely precipitated at about this concentration. There is also some evidence,

particularly in experiment 399, of another break occurring in the neighbourhood of 70 p.c. alcohol. This was quite marked in three of the five experiments done, indicating a complex nature for the more-soluble fraction.

The lower curves represent the same "organic" fraction after incubation. There is no significant change noticed due to the addition of glucose in contrast to tumour. It is also evident that the increase in free P during incubation (apart from the creatine P) is derived from at least part of both the more-soluble and the less-soluble fractions. By comparing the acid hydrolysis of extracts of rabbit muscle before and after incubation Davenport and Sacks [1929] likewise found this "enzyme hydrolysable" fraction behaved as at least two different substances. It is not possible from Ba precipitation experiments to deduce the exact amount of free P contributed by either fraction. The liberation of (more-soluble Ba-) adenylic acid from (less-soluble Ba-) adenylpyrophosphate (Lohmann) would involve an increase in the more-soluble fraction with a decrease in the less-soluble, without, *per se*, involving any liberation of free P. In Eggleton's experiment with oxygen lack on muscle a time relationship is noted between the disappearance of the pyro-P and "insoluble" ester, and the increase in the "soluble" ester. In the minced muscle there is definitely some liberation of free P from the pre-existing more-soluble fraction.

Discussion.

From these results on C 180 mouse tumour and minced muscle certain comparisons may be made. The lactic acid produced in 1 hour by minced muscle without sugar is much larger than that produced by the surviving tumour under similar conditions. Several authors are agreed that tumour contains a comparatively large quantity of glycogen. Presumably this cannot undergo glycolysis to the same extent as in muscle.

The free P of the tumour is of the same order as that found for resting muscle which is in diffusible equilibrium with a free P concentration similar to that in plasma. In contrast to the muscle there is only a very small quantity, if any, of labile P present in the tumour. By comparing the fractional precipitation curves it is evident that the "organic" acid-soluble P which exists in comparatively small quantities in tumour does not behave similarly to that of minced muscle during incubation. By comparing the behaviour of the tumour incubated in the presence and absence of added glucose it is seen that the liberation

of free P bears a certain reciprocal relation to the formation of lactic acid and in this respect behaves similarly to the liberation of ammonia as shown by Warburg.

While this paper was being reported, further work on phosphate metabolism of tumour has been published by Edlbacher and Kutscher [1931]. Their finding as to the incidence of creatine P is essentially in agreement with ours for C 180 tumour. They also confirm the statement of Barr, etc. [1928] that fluoride is without influence on the free P changes in the incubated tumour. Fluoride also inhibits glycolysis. This may otherwise increase the free P liberation similar to the absence of sugar, masking a characteristic fluoride effect of removing free P. Edlbacher and Kutscher's finding that tumour can liberate free P from nucleic acid probably accounts for at least part of the increase in the acid-soluble P found by us. They also found some hydrolysis of hexose-diphosphate by minced tumour. It is interesting to note that in all cases where preparations of tumour have successfully attacked a hexosephosphate [Neuberg, Downes, Edlbacher], either the magnesium salt or a solution containing magnesium has been used. Magnesium is known to be an essential element in coferment of muscle and yeast.

Summary.

1. There is a trace of labile (creatine phosphorus) present in C 180 mouse tumour.

2. The increase in free phosphate of the tumour extracts on incubation is greater in the absence of added glucose than when glucose is present in the incubating fluid, thus showing a certain reciprocal relation to glycolysis.

3. During incubation of the mouse tumour there is an increase in acid-soluble phosphate. This is derived from the non-lipoid phosphorus of the precipitate. Only in the absence of glucose is the free phosphate liberated large enough to account for all of the new acid-soluble phosphate. There is thus an accumulation of an acid-soluble organic phosphate during incubation of the tumour in the presence of glucose when glycolysis is large.

4. From fractional precipitation of barium salts by alcohol no evidence is obtained of a shift from a hexosemonophosphate to hexose-diphosphate during glycolysis of surviving tumour. The organic phosphate which accumulates only in the presence of glucose is precipitable as a single fraction by 20 p.c. alcohol in presence of Ba.

5. The "enzyme hydrolysable" phosphate of minced mouse muscle consists of at least two separate fractions. There is some evidence that the more-soluble may be further differentiated.

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