THE INCORPORATION OF ^{32}P-LABELLED ORTHOPHOSPHATE INTO NUCLEOTIDES*

by**

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In an actively metabolizing system the incorporation of labelled orthophosphate into nucleotides, and other phosphorylated compounds, may be considered as two separate processes. In the first, if the system is exposed to phosphate for a relatively long period of time, it is to be expected that all phosphorylated compounds ultimately will become labelled owing to the dynamic state of their existence. In this state of continuous breakdown and resynthesis the rate of labelling of a particular substance, and the time required for complete equilibration, is a function of the turnover rate. Such studies have been documented in detail by HEVESEY[1] and KAMEN[2], while more recently, CORI *et al.*[3] and ZETTERSTRÖM AND LJUNGGREN[4] have utilized this property to biosynthesize ^{32}P-labelled DPN of high specific activity.

The second type of labelling is realized in a much shorter period. It is believed to be connected with the process of oxidative phosphorylation, the essential features of which may be summarized as follows: during the oxidation of certain substrates in, and adjacent to, the citric acid cycle, the energy is trapped and stored for future use through the medium of high energy phosphate bonds. This is accomplished by orthophosphate being esterified during the reversible oxidation-reduction of the coenzymes which comprise the electron transport system between the substrate and the oxidant[5,6,7]. Such "coenzyme phosphates" are assumed to be both transient and labile in order to account for the present inability to achieve their isolation. In the intact system, however, these "coenzyme phosphates" may transfer their high energy phosphate to more stable substances, the first step being envisioned as the synthesis of ATP from ADP or AMP.

If this hypothesis is correct, oxidative phosphorylation will result in all of the

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participating nucleotides being labelled, at least transiently, after short exposures to ^{32}P-labelled orthophosphate. Upon isolating the nucleotides, however, labelling would be found only in those wherein the phosphate had formed a stable linkage.

It is difficult to obtain unequivocal proof that a given nucleotide is labelled since most of these substances cannot be isolated in pure form, and recourse must be made to inferential experiments where the nucleotide and radioactivity are shown to accompany each other throughout various purification procedures.

LINDBERG and his colleagues, in an extensive and systematic series of investigations[4,8–11], have directed attention toward this latter type of nucleotide labelling. In addition to their theoretical contributions[12], this group has also developed many useful techniques and methods[13,14] related to the problems of phosphorylation. Previous work in our laboratory has been concerned with certain aspects of oxidative phosphorylation[15–18] including the semi-quantitative fractionation by chemical methods of ^{32}P-labelled substances generated in actively metabolizing cyclophorase preparations[19]. The present investigation[20] is an extension of this problem, namely, the more complete identification of the ^{32}P-labelled nucleotides.

RESULTS AND DISCUSSION

Chemical fractionation

Initially, a study was made of the level of nucleotides and other phosphorus-containing compounds in cyclophorase preparations and their behaviour upon chemical fractionation of the type employed by ALBAUM[19]. The original fractionation scheme has been reduced to two simple classifications: barium-insoluble and ethanol-insoluble, the details of which are given in the Experimental section. Within these fractions, the occurrence of various nucleotides has been ascertained with the aid of paper chromatography and by means of specific microbiological and enzymic assays.

The *absolute* amount of any particular nucleotide, on a dry-weight basis, varied with different liver preparations (R_3L). Within a given preparation, however, the *relative* amounts of different nucleotides were approximately constant and showed a consistent behaviour upon fractionation. Because of the above variations, the absolute amounts are omitted and only the following qualitative observations are presented: Essentially all of the P_i*, P_7, flavins and ATP were found in the barium-insoluble fraction, while vitamin B_6, thiamine, niacin, DPN, TPN and AMP were almost completely in the ethanol-insoluble fraction. Less sharp separations were noted for ADP and CoA, both of which favoured slightly the former fraction while P_0 and pantothenic acid were in slight, to moderate, excess in the latter fraction. It should be noted further that more ADP was found than ATP (see also Table V) and that the amount of CoA was roughly equal to that of the adenosine polyphosphates. Inositol and biotin were found in trace amounts only in the ethanol-insoluble fraction. No major differences were found in the above picture when kidney cyclophorase (R_3K) replaced liver, or when the liver preparations were incubated with substrate at 5° instead of 38°.

Although it is well-known that much of the variation in absolute level of the

* The following abbreviations will be used: Inorganic phosphate (P_i), 7-minute phosphate (P_7), 100-minute phosphate (P_0), adenosine mono-, di- and triphosphates (AMP, ADP, ATP), coenzyme A (CoA), diphosphothiamine (DPT), di- and triphosphopyridine nucleotide (DPN, TPN), flavin-adenine dinucleotide (FAD), an unknown flavin dinucleotide (FAD–X), flavin mononucleotide (FMN) and riboflavin (Rb).

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nucleotides is due, simply, to the inherent variation of the individual animals, a part of this difficulty is caused by the incompleteness of the precipitation steps. Furthermore the multiplicity of compounds within a given fraction, and the appearance of certain compounds in both fractions were serious disadvantages in the use of these methods. As an example, it was found that CoA, contained in the barium-insoluble fraction, is only partially brought into solution upon treatment with acid, as shown in Table I.

TABLE I

LIBERATION OF CoA BY ACID TREATMENT OF BARIUM CoA

Fraction	*Total weight (mg)*	*Units CoA/mg*	*Total Units CoA*
Acid insoluble	282	0.62	175
Acid soluble	112	1.7	190

More extensive schemes of chemical fractionation, such as originally used by ALBAUM[19] or those devised by LE PAGE[21] and SACKS[22], did not appear to offer a solution to this fundamental problem of *unequivocally* assigning ^{32}P-labelling to a given nucleotide. Accordingly, attention was turned toward chromatographic methods which are capable of giving more complete separations.

Paper chromatography, in particular, has been employed previously in similar investigations[9], and was used, likewise, throughout the course of this problem. The techniques employed, both with regard to the running of the chromatograms and the detection of the various substances are described in the Experimental Section. The R_F values of authentic samples in the different solvent systems are listed in Table II.

TABLE II

R_F VALUES OF COMPOUNDS

Compound	*5% Na_2HPO_4**	*Collidine***	*Phenol***	*Phenol/n-butanol***
FAD	0.35	0.17	0.57	0.20
FMN	0.45	0.04	0.40	0.17
FAD–X	0.35	0.30	—	0.41
Rb	0.28	0.69	0.63	0.79
Lumiflavin	0.18	0.85	0.95	0.94
Lumichrome	0.07	0.72	—	0.88
AMP	0.70	0.05	0.32	0.08
ADP	0.75	0.00	0.20	0.03
ATP	0.80	0.00	0.10	0.00
DPN	0.72	0.06	0.74	0.32
TPN	0.85	0.01	0.44	0.08
CoA (S–S form)	0.95	0.09	0.66	0.02
P_i	0.95	0.07	0.12	—

* The R_F values in this solvent system are markedly dependent upon the numerous variables encountered in paper chromatography: presence of other salts in the sample, temperature, pH, etc.
** Organic phase saturated with H_2O.

Further characterization of the barium- and ethanol-insoluble fractions, mentioned above, was accomplished by chromatographing them in the phosphate buffer system.

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The results are illustrated in Fig. 1. The radioactive areas were determined both by radioautographs and by direct counting of the segmented paper, as shown.

In the barium-insoluble fraction the fluorescent spots at R_F values of 0.18, 0.35 and 0.43 are attributed to lumiflavin, FAD and FMN, respectively, and are not labelled. The quenching spots at 0.67 and 0.74 correspond to ADP and ATP and both appear to be labelled. No quenching or fluorescent spots are visible in the region of 0.95 where considerable radioactivity is found. CoA may be detected enzymically in this area, but is in too low a concentration to register as a quenching spot. However, the CoA cannot be designated unequivocally as being labelled since orthophosphate (P_i) is known to migrate to the same region. The streaking of radioactivity between 0.2 and 1.0, although with evident peaks, appears to be caused by the introduction of excess ions during the ecomposition of the barium precipitate. In contrast, the ethanol-insoluble fraction hadsonly two distinct peaks.

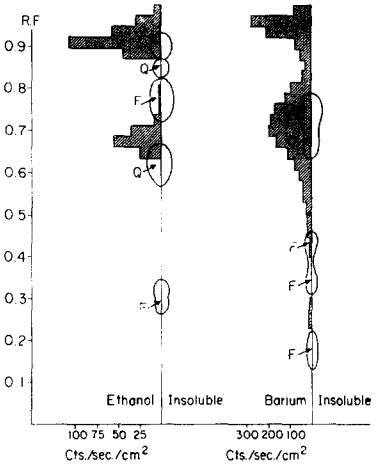

Fig. 1. Paper chromatograms of ethanol- and barium- insoluble fractions. Aliquots of the barium-insoluble and ethanol-insoluble fractions, as described in the text, were chromatographed in the phosphate buffer system. Fluorescent (F) and quenching (Q) spots were located by inspection under the ultraviolet lamp, while the radioactivities were measured by cutting the strip into small segments and counting each directly.

In the ethanol-insoluble fraction the fluorescent spot at 0.30 corresponds to riboflavin which arises during isolation and chromatography as a breakdown product of FMN and FAD[23]. The quenching spot at 0.62 is AMP and does not appear to be labelled. The radioactive area at 0.68 may be due to ADP in this fraction in too small a quantity to be detected visually as a quenching spot. The fluorescent-quenching-fluorescent triad at 0.77, 0.85 and 0.90 is characteristic of crude CoA fractions (unpublished observations in this laboratory) and is displaced downward on the paper probably because of the presence of high salt concentrations in the sample. The radioactivity at 0.90 coincides with the uppermost of these spots.

Chromatographic fractionation

Previous studies have demonstrated that several classes of nucleotides may be separated by means of adsorption chromatography on Florisil columns[23]. Even with the smaller amounts of nucleotides available from a single rabbit liver, the method proved to be applicable. In a typical experiment, whose details are described more fully in the Experimental Section, a liver *homogenate* was allowed to oxidize L-glutamate in the presence of ^{32}P-orthophosphate, and the nucleotides were examined for radioactivity. Including the initial TCA effluent, five fractions were obtained, as shown in the flow diagram, Fig. 2, which lists, also, some of the principal constituents in each eluate. The recovery of radioactivity in such an experiment is shown in Table III. Essentially all of the radioactivity is TCA-soluble (*cf.* 2 with 1) and very little of this radioactivity is lost on the columns (*cf.* 10 with 2). The radioactivity in eluates III, IV and V is due, largely, to nucleotides which have become labelled during the oxidative processes. As

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shown in Table IV, these fractions contain significantly larger amounts of radioactivity in the experimental run as contrasted to the two blank runs which were identical except that in one blank the homogenate was maintained under anaerobic conditions, and in the other, the labelled phosphate was added *after* deproteinization. It is of interest that

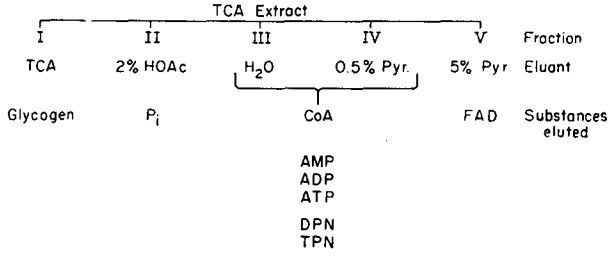

Fig. 2. Flow diagram for florisil column.

the "nucleotide radioactivity", which may be defined as the sum of the counts in eluates III, IV and V, is 8.4 times larger in the experimental run than in the anaerobic blank. In a similar type experiment where the TCA extracts were compared, ALBAUM[19] found 9.1 times as much labelling in the experimental sample as in the blank.

TABLE III

RADIOACTIVITY IN CHROMATOGRAPHIC FRACTIONATION

No	*Fraction*	*Total Radioactivity counts/min*
1	$H^{32}PO_4^{-2}$ added initially	$2.29 \cdot 10^8$
2	TCA extract	$2.56 \cdot 10^8$
3	Protein residue	$2.60 \cdot 10^6$
4	Sum: 2 and 3	$2.58 \cdot 10^8$
5	TCA effluent (I)	$3.67 \cdot 10^7$
6	2% HOAc eluate (II)	$1.39 \cdot 10^8$
7	H_2O eluate (III)	$1.76 \cdot 10^7$
8	0.5% Pyr. eluate (IV)	$3.60 \cdot 10^6$
9	5% Pyr. eluate (V)	$7.02 \cdot 10^5$
10	Sum: 5, 6, 7, 8 and 9	$1.99 \cdot 10^8$

TABLE IV

COMPARISON OF EXPERIMENTAL AND BLANK RUNS

Fraction	*Experimenta*	*Blank**	*Blank***
III	$1.76 \cdot 10^7$	$2.14 \cdot 10^6$	$5.62 \cdot 10^6$
IV	$3.60 \cdot 10^6$	$4.39 \cdot 10^5$	$0.65 \cdot 10^5$
V	$7.02 \cdot 10^5$		$1.84 \cdot 10^5$
Sum III, IV, V	$21.9 \cdot 10^6$	$2.6 \cdot 10^6$	$0.5 \cdot 10^6$

* Gassed with N_2 ** $H^{32}PO_4^{-2}$ added after deproteinization.

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Adenine nucleotides

The adenine nucleotides and CoA, contained in fraction III, were concentrated and separated from each other by a combination of chemical and chromatographic techniques. The nucleotides were collected from the eluate by barium-acetone precipitation and the barium precipitate divided into two parts. The first, IIIa, was freed from barium, placed on an ion-exchange column, and the adenine nucleotides separated quantitatively according to the method of COHN AND CARTER[24]. The elution data and the expected constituents of each fraction are given in Table V. For each fraction the per cent of total adenine and total radioactivity are calculated on the basis of the total *actually eluted from the column.* In terms of the amounts originally placed on the column, only 74.5 and 82.3% of the adenine and radioactivity, respectively, were recovered. Unsuccessful attempts were made to elute the material remaining on the column after fraction 5 using solvents up to 50% acetic acid. Taking radioactivity as a guide, this tightly-bound material could be eluted slowly if the column was allowed to stand in contact with 1 *N* HCl.

TABLE V

FRACTIONS FROM DOWEX-1 COLUMN

Fraction	*Component*	*% of Total**	
		260 mμ Absorption	*Radioactivity*
1	Adenosine	2.8	8.8
2	Adenine	0.0	1.7
3	AMP	82.5	47.6
4	ADP, Pi	12.8	36.5
5	ATP, PP	1.9	5.4
Recovery %		74.5	82.3

* Based upon Total Amount *eluted* from column.

The above evidence indicates that a bound nucleotide was being hydrolyzed slowly in acid to a smaller fragment which was elutable under acid conditions. This hypothesis is strengthened by the observation that AMP, as judged by paper chromatography, is the fragment finally discharged from the column. It is known that other nucleotides, such as CoA, DPT, DPN and TPN are adsorbed on, and not elutable from a Dowex-1 column under similar conditions; other experiments described in this paper, however, show that the pyridine nucleotides are unlabelled and cannot account for the residual activity on the column.

The bound, labelled nucleotide might also be an adenosine polyphosphate. Compounds of this type have been suggested in other investigations[12], and, in fact, ALBAUM[19] obtained a labelled fraction (No. 6 in his nomenclature) which was devoid of AMP but had ADP and ATP-like activity in the hexokinase-myokinase-deaminase test system. This material could not have been ADP or ATP, as such, since the barium salt was both acid-insoluble and only partially brought into solution by treatment with sodium sulfate. It should be noted that in this investigation when fraction IIIa was originally prepared from the barium salt of fraction III (see above), not all of the radioactivity went into solution, but was gradually removed by repeatedly washing the mixed barium precipitate (barium sulfate and barium nucleotides) with acid.

From Table V it is seen that in fraction IIIa the relative amounts of the adenine

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nucleotides are: AMP > ADP > ATP. Since the adenine nucleotides are found also in the 0.5% pyridine eluate (fraction IV), the amounts here do not represent, of course, the relative levels or amounts existing in the original homogenate, although the relative order is consistent with other findings[25]. No explanation can be advanced as to the nature of the radioactivity in the AMP fraction, since other experiments, mentioned previously in this paper and results of other investigations[9,19] have shown that AMP is not labelled. Recently, BARTLETT[26] has separated other phosphorylated compounds by means of a Dowex-1 column; in the fraction considered above, glucose-6-phosphate is a constituent. Furthermore, since SACKS[27] has shown that glucose-6-phosphate, as well as glucose-1-phosphate, readily incorporates radioactive phosphate, the possibility exists that at least a portion of the radioactivity in the AMP fraction may be due to hexose phosphate.

Coenzyme A

The remainder of the water eluate, fraction IIIb, was worked up by a procedure designed to separate CoA from the adenosine phosphates. Dinucleotides such as DPN, TPN, FAD and CoA may be extracted into phenol from a water solution saturated with ammonium sulfate, while AMP, ADP and ATP are left, largely, in the water phase. Accordingly, fraction IIIb was freed from barium with sodium sulfate and the nucleotide taken into phenol and returned to water. This fraction was shown to contain CoA by enzymic analysis and an aliquot chromatographed on paper in the phosphate buffer system. A single "quenching" spot was observed at R_F 0.95, which gave a positive S–S reaction when sprayed with nitroprusside-cyanide[28] or iodine-azide[29]. A radioautograph showed that this spot was labelled. Since orthophosphate likewise migrates to R_F 0.95 in this solvent system, the above evidence is not conclusive that the CoA is ^{32}P-labelled. However, reference to the original phenol extraction yields additional information. A "partition coefficient", K, may be defined as the ratio of material extracted into the phenol phase relative to that remaining in the aqueous phase. Using the radioactivity as an indicator, K is equal to $76 \cdot 10^{-3}$. Although very little of the radioactivity was phenol-soluble it is indicative, nonetheless, of a radioactive nucleotide since K, in the case of orthophosphate carried through the identical procedure, was found to be much smaller, namely, $5 \cdot 10^{-3}$.

The above evidence does not permit a distinction to be made between labelling in CoA, as such, and a CoA derivative, possibly S-phosphoryl CoA[30]. Obviously, the final decision as to whether CoA or one of its derivatives is, or is not, labelled must await further work, possibly employing on a small scale the methods recently described for the isolation of highly purified CoA[31,32]. It should be mentioned also that during this investigation no attempt was made to control the multiple oxidation-reduction forms which CoA may assume[33–35]. As isolated by the above procedures, the CoA appeared to be in the disulfide form since it did not react with nitroprusside until treated with cyanide.

Flavin nucleotides

Fraction V, the 5% pyridine eluate from the Florisil column, contained the flavin nucleotides, and, as shown in Table IV, only a relatively small amount of radioactivity. The fraction was concentrated and chromatographed on a partition column of phenol-butanol/water with Celite as the supporting phase according to the method of DIMANT

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et al.[23]. FAD and FAD–X were collected separately and found to be devoid of radioactivity (see Table VI). The yield data, given also in Table VI for these flavins, is comparable to that obtained from larger batches of rabbit liver. No riboflavin or flavin mononucleotide (FMN) was found in the tissue in agreement with previous results from this laboratory[23] and elsewhere.

TABLE VI

ISOLATION OF FLAVIN NUCLEOTIDES

	Total Cts.	*Total Flavin* (μg)	*R value**
Fraction V	$7.02 \cdot 10^5$	906	5.7
FAD	0	458	6.9
FAD–X	0	76	4.5

Recovery of flavin after partition chromatography = 60%.

* Ratio of light absorption at 260 and 450 mμ.

Phenol-soluble nucleotides

In their investigation of this same problem, HUMMEL AND LINDBERG[9] chromatographed on paper the "phenol-soluble" nucleotides and determined in a semiquantitative manner the coincidence of radioactivity with the various nucleotides. Since this technique is capable of affording considerable information, especially concerning the *absence of label* in certain nucleotides, it was employed likewise in this investigation.

As a preliminary experiment, a rabbit liver homogenate was prepared and treated exactly according to the directions of HUMMEL AND LINDBERG. Aliquots of the "phenol-soluble" nucleotide fraction were chromatographed on paper using certain of their solvent systems and several additional ones. Substances were assumed to be radioactive only if the shape of the radioautograph spot coincided with the visually observed spot.

The results with three of the solvent systems employed by HUMMEL AND LINDBERG, (a) butanol/acetic acid/water, (b) butanol/phenol, and (c) pyridine) are not given, since, in agreement with their findings the nucleotides are not moved beyond R_F 0.10 in the first two systems, while in the latter case, the residual pyridine, even after repeatedly washing the paper with chloroform, obscures the quenching and fluorescent spots.

In collidine/water three radioactive areas are observed, the lowest one being less intense than the upper two. This is in agreement with the pattern observed by HUMMEL AND LINDBERG except that their spots have much higher R_F values—a finding which is not surprising since collidine from different sources is known to show variations in chromatographic behaviour. Direct observation of the paper revealed only one yellow fluorescent spot (FAD) corresponding to the upper radioactive spot. The second spot may be due to ATP which migrates near that area while the lowest spot was too near the origin to be identified.

With phenol/water only one quenching spot could be observed visually, and this coincided with the radioactivity. No second radioactive spot, lower on the paper, was detected, contrary to the findings of HUMMEL AND LINDBERG. None of the flavins has an R_F value in this system corresponding to the radioactivity.

That FAD is not labelled is demonstrated further by chromatography in Na_2HPO_4. This only radioactive area is near the solvent front corresponding to the known location

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of CoA, while FAD is far below. Furthermore, in the system phenol/butanol water, the radioactivity moves in two well-defined areas between the origin and R_F 0.10, the lower spot corresponding to a blue-fluorescing substance of unknown identity, while FAD is clearly seen to be unlabelled as is also the faint spot of FAD-X.

It has been confirmed that in two of the solvent systems employed by HUMMEL AND LINDBERG, the radioactivity and FAD migrate to the same area; however, by an extension of this study to other solvents the radioactivity and FAD may be separated and additional evidence has been obtained that CoA is at least one of the labelled nucleotides in the phenol-soluble group. It seems doubtful that an additional flavin nucleotide containing a transient, labile phosphate bond need be invoked[12] to explain the apparent coincidence of label with flavin in some instances, since such a labile substance would probably not survive the isolation procedures used in this investigation and elsewhere. Furthermore, while several new flavin nucleotides have recently been described[36,37,38], all seem to possess normal phosphate linkages comparable to FAD or FMN.

EXPERIMENTAL

Materials

AMP, ADP, ATP, DPN, TPP, FMN and the various vitamins were commercial samples. FAD was prepared by the method of DIMANT *et al.*[23], while purified CoA concentrates were obtained by several different procedures[31,32,39] or were generously supplied by Dr. F. M. STRONG or the Upjohn Co. ^{32}P-orthophosphate was obtained from the United States Atomic Energy Commission, Oak Ridge. Florisil, a magnesium silicate adsorbent (30/60 mesh) was a product of the Floridin Co., Warren, Pennsylvania.

Methods

AMP, ADP and ATP were determined by the hexokinase-myokinase-deaminase method of KALCKAR[40]; DPN and TPN by alcohol dehydrogenase[41] and isocitric dehydrogenase[42], respectively, FAD by D-amino acid oxidase[43] and CoA by acetylation of sulfanilamide[44]. The vitamins were assayed for by conventional microbiological methods. The procedure recommended by KING[45] was employed for the estimation of P_i, P_7 and P_0.

Chemical fractionation studies

Cyclophorase preparations of rabbit liver and kidney R_3L and R_3K were obtained by the methods previously described from this laboratory[46,47]. The protocol for exposing the preparations to ^{32}P-labelled orthophosphate was essentially the same as that described by ALBAUM[19].

The reaction mixture was deproteinized by adding an equal volume of cold 10% TCA and the supernatant recovered by centrifugation in the cold at 4000 × *g* for 15 minutes. All subsequent operations were likewise carried out in the cold (5° C). The residue was extracted once again with 20 ml of 5% TCA and the supernatants pooled and adjusted to pH 8.2, 0.1 volume of 20% barium acetate was added, and after standing 20 minutes, the precipitate was recovered by centrifugation and the supernatant retained for subsequent use. The precipitate was dissolved in 0.1 *N* HCl and reprecipitated by adjusting the pH to 8.2 and adding one drop of barium acetate. The precipitate was again dissolved in 0.1 *N* HCl and 0.2 *M* sodium sulfate was added in small portions to precipitate Ba^{+2}. Excess sulfate was avoided. After removal of the barium sulfate by centrifugation, the supernatant (barium-insoluble fraction) was neutralized to pH 7.0 and lyophilized.

The supernatant from the initial barium precipitation (pH 8.2) was treated with 4 volumes of cold 95% ethanol and, after standing for 30 minutes, the precipitate was recovered by centrifugation. The precipitate was dissolved in 0.1 *N* HCl and the resulting solution neutralized and lyophilized (ethanol-insoluble fraction).

Chromatographic fractionation studies

Rabbit liver homogenates were prepared and, in the experimental run, were exposed to ^{32}P-orthophosphate (about 10^8 counts/min) exactly according to the directions of HUMMEL AND LINDBERG[9]. In addition, a 1.0 ml aliquot of the homogenate was checked manometrically for active oxidation under conditions existing in the large-scale experiment, and, although the rate of oxidation was low for homogenates, due to the high level of fluoride present, it was sufficient to insure an adequate turnover of the participating nucleotides.

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Blank runs were also made under conditions identical with those above except that in the first blank the homogenate was gassed with 95% N_2 — 5% CO_2 instead of O_2 CO_2, while in the second blank the radioactive orthophosphate was added after TCA deproteinization.

After deproteinization, the TCA extract was passed through a column of Florisil (3.5 × 17 cm), and the adsorbed nucleotides washed, in order, with 2 liters of 5% acetic acid, 3 liters of water, and 2 liters of 0.5% pyridine. One hundred ml of 5% pyridine removed the flavin from the column, completing the chromatography.

Two liters of the water eluate, fraction III, from the Florisil column were adjusted to pH 8.5, 20 ml of 2 *M* barium acetate added, followed by 4 liters of cold acetone, and, after standing overnight at 5° C the flocculent precipitate was collected first by siphoning off the excess liquid and then centrifuging. After washing with cold acetone and ether and drying *in vacuo*, it was obtained as a white powder (5.2 g).

For ion-exchange chromatography, an aliquot (1.8 g) of the above barium precipitate was thoroughly mixed in the cold with 0.9 g of anhydrous sodium sulfate, 5 ml of H_2O and sufficient 5% sulfuric acid to bring the pH to 3.0. The supernatant was recovered by centrifugation, whereupon the residue was washed once with 2 ml of dilute sulfuric acid (pH 3.0). After the pooled supernatants, pale yellow in color, had been adjusted to pH 8.5 the nucleotides were precipitated with 9 volumes of cold acetone, washed with acetone and ether and dried *in vacuo*. The white powder was dissolved in 2.0 ml of 1 *M* NH_4OH and chromatographed on a 1 × 2 cm. Dowex-1 column (OH^- form, 8% cross-linked) using the method of COHN AND CARTER[24]. For each eluting fraction 10 ml aliquots were collected until both the "adenine" material, determined by light absorption at 260 mμ, and the radioactivity had declined to zero or asymptotically to low values. In Table V the total adenine and radioactivity are summed over each fraction.

Paper chromatography

All paper chromatograms were run in one direction (ascending — with the solvent front travelling about 30–35 cm) on sheets of Whatman No. 1 paper. The solvent systems employed were: 5% Na_2HPO_4[48], phenol/butanol/H_2O[23], phenol/H_2O[9] and collidine/H_2O[9]. After drying, the papers were examined under ultraviolet light*; the flavins were located by means of their intense yellow fluorescence while adenine-containing compounds were detected as dark blue "quenching" spots. When pyridine or phenol were used as solvents, it was necessary to wash the papers repeatedly with chloroform in order to see the fluorescing or quenching spots. Phosphorylated compounds were identified by the molybdate spraying method of AXELROD AND BANDURSKI[49], SH compounds, such as CoA, by the nitroprusside[28] or iodine-azide sprays[29], and the pyridine nucleotides by the methyl ethyl ketone — ammonia method[50].

In certain experiments the spots were eluted from the paper and the contents estimated enzymically or counted for radioactivity (see Fig. 1). It should be noted in this connection that CoA as a spot on a paper chromatogram cannot be kept at room temperature without losing its enzymic activity: 75% of the original activity remains after 1 day while only about 50% remains after 3 days. When stored at —20° C, however, the entire activity was preserved for periods up to 10 days.

R_F values given in Table II are *averages* of numerous determinations. Since R_F values vary with many factors (*e.g.*, temperature, contaminating salts, time of equilibration, age of the solvent system, *etc.*), all identifications referred to in this paper were made using internal standards on the same paper.

Radioautographs were made by exposing the paper chromatograms to No-Screen X-ray film for periods of 1–3 days.

Radioactivity measurements

All radioactive samples were determined by plating 20 or 50 λ aliquots on thin aluminum planchets, which were then counted with a Berkeley Auto Scaler and corrected for the small background.

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* "Mineralight" lamp, Model V-41, supplied by Ultra-Violet Products Corporation, South Pasadena, California.

References p. 423/424.

SUMMARY

1. Chemical fractionation, column and paper chromatographic methods have been employed to study the short-term incorporation of ^{32}P-orthophosphate into various nucleotides and other phosphorylated compounds in respiring rabbit liver homogenates.

2. No evidence could be obtained for the labelling of flavin or pyridine nucleotides. AMP does not appear to be labelled, in contrast to ADP and ATP which are labelled. CoA, or one of its derivatives, is probably labelled.

3. An unidentified compound, possibly an adenosine polyphosphate, is labelled.

RÉSUMÉ

1. L'incorporation à court terme de ^{32}P-orthophosphate dans divers nucléotides et autres composés phosphorylés d'homogénats de foie de lapin en aérobiose a été étudiée par fractionnement chimique, et par chromatographie sur colonne et sur papier.

2. Le marquage des flavine et pyridine nucléotides n'a pu être mis en évidence. AMP ne semble pas être marqué; au contraire ADP et ATP le sont. CoA, ou l'un de ses dérivés, est probablement marqué.

3. Un corps non identifié, peut-être un adénosine polyphosphate, est marqué.

ZUSAMMENFASSUNG

1. Chemische Fraktionierung, Säulen- und Papierchromatographische Methoden wurden zur Untersuchung des Kurzzeit-Einbaues von ^{32}P-Orthophosphat in verschiedene Nucleotide und andere phosphorylierte Verbindungen in respirierenden Kaninchenleberhomogenaten verwendet.

2. Es konnte kein Beweis für die Markierung von Flavin- oder Pyridinnucleotiden erhalten werden. AMP scheint nicht markiert zu werden im Gegensatz zu ADP und ATP, die markiert werden. CoA oder eines seiner Derivate wird wahrscheinlich markiert.

3. Eine nicht identifizierte Verbindung, möglicherweise Adenosinpolyphosphat wird markiert.

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